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Actin dysfunction in neurodegenerative disorders:

Uncovering Cofilin-1 pathology in Lewy Body dementias

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ACTIN DYSFUNCTION IN NEURODEGENERATIVE DISORDERS: Uncovering Cofilin-1 pathology in Lewy Body dementias

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Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less. — Marie Curie

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CONTENTS

ABSTRACT

Neurons are peculiar cells with extremely long processes where the cytoskeleton is crucial not only for the proper neuronal development but also for a sustained function of the nervous system. Thus, the maintenance of a proper cytoskeleton architecture and function is critical to preserve neuronal functionality. One of the main cytoskeleton components is actin, and alterations of the actin cytoskeleton and its molecular regulators have been increasingly reported in different neurodegenerative disorders. This proposes that actin cytoskeleton damage might be a common feature of several unrelated neurodegenerative diseases. This idea prompted us to further dissect actin cytoskeleton alterations and the underlying molecular mechanisms in the context of neurodegeneration, specifically resultant from prone-to-aggregate proteins.

This thesis started with a study in the context of Familial Amyloid Polyneuropathy (FAP), a fatal autosomal dominant disease caused by the extracellular deposition of amyloid fibrils containing mutated transthyretin (TTR), particularly in the peripheral nervous system, ultimately leading to neuronal death. Using a *Drosophila* model for the disease, we discovered that actin cytoskeleton alterations occur in FAP, being triggered by members of the Rho GTPase signaling pathway, a major actin regulator. This prompted us to study actin cytoskeleton-related defects in other neurodegenerative diseases, namely in synucleinopathies.

Synucleinopathies are neurodegenerative disorders arising from massive α-Synuclein (αSyn) accumulation and comprise the second most common cause of neurodegeneration in the world. Cognitive impairment is a prominent feature of these diseases that has been overshadowed by the motor dysfunction, typical of Parkinson's Disease (PD). When cognitive impairment and dementia are present, these disorders are designated Lewy Body dementias (LB dementias) and include Parkinson's disease dementia (PDD) and Lewy Body dementia (DLB). Lewy Body (LB) pathology, characterized by αSyn-containing aggregates, determines the neuropathologic staging of the disease at autopsy, and there is a correlation of increased hippocampal and cortical LB pathology with decreased cognitive performance in LB dementias. In fact, increased αSyn aggregation in the hippocampus is associated with neuronal dysfunction, and hippocampal volume loss was observed in LB dementia patients. Altogether, the current knowledge points to the hippocampus as one of the most vulnerable regions affected by αSyn pathology. Since the hippocampus is a central brain region involved in learning and memory, and considering the described observations in LB dementia patients, it is expected an impact of αSyn on the

hippocampus underlying synaptic impairment and cognitive decline present in these patients.

The pathologic consequences of αSyn accumulation in the hippocampal synaptic function as well as the underlying molecular mechanisms remain understudied, and constituted the major focus of this thesis. To address this question, we analyzed the effect of αSyn overexpression or extracellular administration of αSyn pre-formed fibril (PFFs) on hippocampal neurons. The use of an overexpressing system better represents a genetic scenario of the disease, where patients have multiplications of the *SNCA* gene (αSyn encoding gene) and present increased levels of the αSyn protein. While the use of αSyn PFFs preferably characterizes the sporadic disease, as the endogenous levels of αSyn are unaltered, and the αSyn PFFs serve as the trigger for αSyn aggregation. Nevertheless, both systems are suitable to study αSyn-induced hippocampal pathology and the results obtained with each model are not mutually exclusive. Here we report that both, αSyn overexpression and αSyn PFFs, induced the dysregulation of the actin-binding protein cofilin leading to its assembly into cofilin-actin rods, which are neuropathologic-related structures, which up to this study were mainly studied in the context of hippocampal pathology in Alzheimer's Disease (AD). Specifically, we found that αSyn-induced rod formation occurs in primary cultures of hippocampal neurons, upon αSyn overexpression and incubation with αSyn PFFs, and also *in vivo*, in the hippocampal brain region of the Thy1aSyn mice, a mouse model of αSyn overexpression in the nervous system, and in a mouse model of αSyn PFFs injection. With the objective of finding molecular mechanisms underlying rod formation in response to αSyn pathology, we found the involvement of signaling pathways implicating the cellular prion protein (PrP^C) and NADPH oxidase (NOX), as well as the chemokine receptors CCR5 and CXCR4. Remarkably, we unraveled cofilin pathology as a mediator of αSyn-induced dendritic spine impairment in hippocampal neurons. Importantly, in the Thy1aSyn mice, a synucleinopathy mouse model with cognitive impairment, we validated cofilin dysregulation and synaptic impairment at the same age when cognitive deficits were observed. This proposes cofilin as a novel player on hippocampal synaptic dysfunction triggered by αSyn on LB dementias.

The data presented in this thesis supports the dysregulation of actin cytoskeleton-related proteins in different neurodegenerative disorders, and unravels new molecular mechanisms involved in disease progression that might prompt novel approaches towards therapy in future studies.

SUMÁRIO

Os neurónios são células peculiares com processos extremamente longos onde o citoesqueleto é crucial não só para o desenvolvimento neuronal adequado, mas também para manter a função do sistema nervoso. Assim, uma manutenção apropriada da arquitetura e função do citoesqueleto é crítica para preservar a função neuronal. Um dos principais componentes do citoesqueleto é a actina, e alterações do citoesqueleto de actina e dos seus reguladores moleculares têm sido cada vez mais reportados em diferentes doenças neurodegenerativas. Esta ideia levou-nos a dissecar as alterações do citoesqueleto de actina e os respetivos mecanismos moleculares, no contexto da neurodegeneração, especificamente resultante de proteínas propensas a agregar.

A presente tese iniciou com um estudo no contexto da Polineuropatia Amiloidótica Familiar (PAF), uma doença autossómica dominante e fatal causada pela deposição extracelular de fibras amiloides contendo a proteína transtirretina (TTR) mutada, em particular no sistema nervoso periférico, resultando em morte neuronal. Usando um modelo de *Drosophila* para a doença, descobrimos alterações do citoesqueleto de actina na doença de PAF, sendo estas despoletadas por membros da cascata de sinalização das Rho GTPases, reguladores principais do citoesqueleto de actina. Isto levou-nos a estudar defeitos no citoesqueleto de actina noutras doenças neurodegenerativas, nomeadamente em sinucleinopatias.

As sinucleinopatias são doenças neurodegenerativas que resultam da acumulação de alfa-sinucleína (αSin) e compreendem a segunda causa mais frequente de neurodegeneração no mundo. O comprometimento cognitivo é uma característica prominente nestas doenças que tem sido ofuscado pela disfunção motora, típica da doença de Parkinson (DP). Quando a disfunção cognitiva está presente, estas doenças são designadas demências com corpos de Lewy que incluem a doença de Parkinson com demência e a demência com corpos de Lewy. A patologia com corpos de Lewy, caracterizada por agregados contendo αSin, determina o estadio neuropatológico da doença aquando da autopsia, e existe uma correlação entre o aumento da patologia na região do hipocampo e córtex com uma performance cognitiva diminuída nas demências com corpos de Lewy. De facto, um aumento da agregação da αSin no hipocampo está associado com disfunção neuronal e menor volume do hipocampo em pacientes com demência com corpos de Lewy. Considerando o referido, o conhecimento atual aponta para o hipocampo como uma das regiões do cérebro mais vulneráveis e afetadas pela patologia de αSin. Sendo o hipocampo uma região do cérebro envolvida em aprendizagem e memória, e considerando as observações descritas nos pacientes com demência com

corpos de Lewy, é expectável que o impacto da αSin no hipocampo esteja subjacente à disfunção sináptica e ao comprometimento cognitivo presente nestes pacientes.

As consequências patológicas da acumulação de αSin na função sináptica do hipocampo assim como os mecanismos moleculares subjacentes permanecem pouco estudados, e constituem o principal foco desta tese. Para estudar esta questão, analisámos o efeito da sobreexpressão de αSin ou a adição extracelular de fibras pré-formadas de αSin em neurónios do hipocampo. Um sistema de sobreexpressão representa melhor um cenário de doença genético, em que os pacientes têm multiplicações do gene que codifica para a αSin (*SNCA*) e apresentam níveis elevados da proteína αSin. Enquanto o uso de fibras de αSin caracteriza preferencialmente a doença esporádica, sendo os níveis endógenos da αSin inalterados, e as fibras servem de estímulo para a agregação da αSin. Não obstante, ambos os sistemas são adequados para estudar a patologia do hipocampo induzida pela αSin e os resultados obtidos com cada modelo não são mutuamente exclusivos. Aqui reportámos que ambos, a sobreexpressão e as fibras de αSin, induziram a desregulação da proteína de ligação à actina, cofilina, levando à sua associação em feixes de cofilina e actina, que são estruturas neuropatológicas, que até este estudo tinham sido maioritariamente estudadas no contexto de patologia do hipocampo na doença de Alzheimer. Especificamente, nós descobrimos que a formação dos feixes induzida pela αSin decorre em culturas primárias de neurónios de hipocampo, após a sobreexpressão de αSin e a incubação com fibras de αSin, e também *in vivo*, na região do cérebro do hipocampo do murganho Thy1aSyn, um modelo de murganho com sobreexpressão de αSin no sistema nervoso, e num modelo de murganho de injeção de fibras de αSin. Com o objetivo de descobrir os mecanismos moleculares subjacentes à formação dos feixes de cofilina e actina em resposta à patologia de αSin, nós descrevemos o envolvimento de vias de sinalização implicando o recetor da proteína priónica celular (PrP^C) e da NADPH oxidase (NOX), bem como os recetores de quimiocinas CCR5 e CXCR4. Notavelmente, revelamos a patologia de cofilina como um mediador do dano das espinhas dendríticas induzido pela αSin. De salientar, no modelo animal Thy1aSyn, um modelo de murganho de sinucleinopatias com defeitos cognitivos, validamos a desregulação da cofilina e o dano sináptico na mesma idade em que foram observados defeitos cognitivos. Isto propõe que a cofilina é um novo intermediário na disfunção sináptica do hipocampo despoletada pela αSin em demências com corpos de Lewy.

Os dados apresentados nesta tese suportam a desregulação de proteínas relacionadas com o citoesqueleto de actina em doenças neurodegenerativas diferentes, e revela novos mecanismos moleculares envolvidos na progressão da doença que podem levar a novas abordagens terapêuticas em estudos futuros.

ABBREVIATION LIST

ABP	Actin binding protein
AD	Alzheimer's disease
ADF	Actin depolymerizing protein
ADP	Adenosine diphosphate
Aip1	Actin interacting protein 1
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ApoE	Apolipoprotein E
APP	Amyloid percursor protein
αSyn	α-Synuclein
ATP	Adenosine triphosphate
Aβ	Amyloid β peptide
CaMKII	Calcium/calmodulin-dependent protein kinase II
CAP1	Adenyl cyclase-associated protein 1
CBA	Chicken β actin
CCR5	CC chemokine receptor 5
Cdc42	Cell division cycle 42
ChEI	Cholinesterase inhibitor
CIN	Chronophin
CMT	Charcot-Marie-Tooth
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
CSPα	Cysteine string protein alpha
CXCR4	CXC chemokine receptor 4
DAPTA	D-ala-peptide T-amide
DIV	Days in vitro
DJ-1	Protein deglycase, PARK7
DLB	Dementia with Lewy bodies
Drp1	Dynamin-related protein 1
EGFP	Enhanced green fluorescent protein

ER	Endoplasmic reticulum
FAP	Familial amyloid polyneuropathy
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBA	Glucocerebrosidase
GCI	Glial cytoplasmic inclusion
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
HAND	HIV-associated neurocognitive disorder
HD	Huntington’s disease
HIV	Human immunodeficiency virus
Hsp90	Heat shock protein 90
IF	Intermediate filament
IRES	Internal ribosome entry site
KO	Knockout
LB	Lewy body
LIMK	LIM kinase
LN	Lewy neurite
LRRK2	Leucine-rich repeat kinase 2
LTD	Long-term depression
LTP	Long-term potentiation
MAP	Microtubule associated protein
mEPSC	Miniature excitatory postsynaptic currents
mGLUR5	Metabotropic glutamate receptor 5
MPS	Membrane periodic skeleton
MPTP	1-metil-4-fenil-1,2,3,6-tetraidropiridina
MSA	Multiple system atrophy
MT	Microtubule
MWM	Morris water maze
NAC	Non-amyloid component

NF	Neurofilament
NOR	Novel object recognition
NOX	NADPH oxidase
OFT	Open field test
PAF	Pure autonomic failure
Pak1	P21 activated kinase 1
PD	Parkinson's disease
PDD	Parkinson's disease dementia
PDGFβ	Platelet-derived growth factor β
PFF	Pre-formed fibril
PINK1	PTEN-induced kinase 1
PIP2	Phosphatidylinositol 4,5-bisphosphate
Plk	Polo-like kinase
PrP^C	Cellular prion protein
PSD	Postsynaptic density
PSD-95	Postsynaptic density protein 95
PTM	Post-translational modification
Rac1	Ras-related C3 botulinum toxin substrate 1
RanBP9	Ran-binding protein 9
REM	Rapid eye movement
RFP	Red fluorescent protein
RhoA	Ras homolog family member A
ROCK	Rho kinase
ROS	Reactive oxygen species
SNARE	soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor
SNpc	Substantia nigra pars compacta
SSH1	Slingshot homologue 1
TTR	Transthyretin
ULF	Unit length filament
VGLUT1	Vesicular transporter 1
WT	Wild type

INTRODUCTION

1 Neuronal cytoskeleton: the actin component

Neurons are the longest cells in animals and the precise integrity of their cytoskeleton is essential to maintain its architecture (Neukirchen and Bradke, 2011). In such highly polarized cells, the neuronal cytoskeleton provides not only support to maintain the three-dimensional scaffold of neuronal shape but also the structure to many cellular functions including cell motility, organelle transport and proper directional flow of signal transmission (Neukirchen and Bradke, 2011). Due to the distinct neuronal morphology, the cytoskeleton proteins also contribute to ensure the proper compartmentalized structure composed by the dendrites, the neuronal soma (cell body) and the axon (Figure 1). The neuronal cytoskeleton comprises three filament structures intimately interconnected, namely intermediate filaments (IFs), microtubules (MTs) and actin microfilaments (F-actin) (Neukirchen and Bradke, 2011) (Figure 1). In addition to these core proteins, cytoskeleton architecture is shaped by several monomer/filament-binding proteins which allows the structure to adopt different arrangements with distinct properties according to the spatio-temporal needs of the cell (Fletcher and Mullins, 2010). A more detailed description of the distribution of the

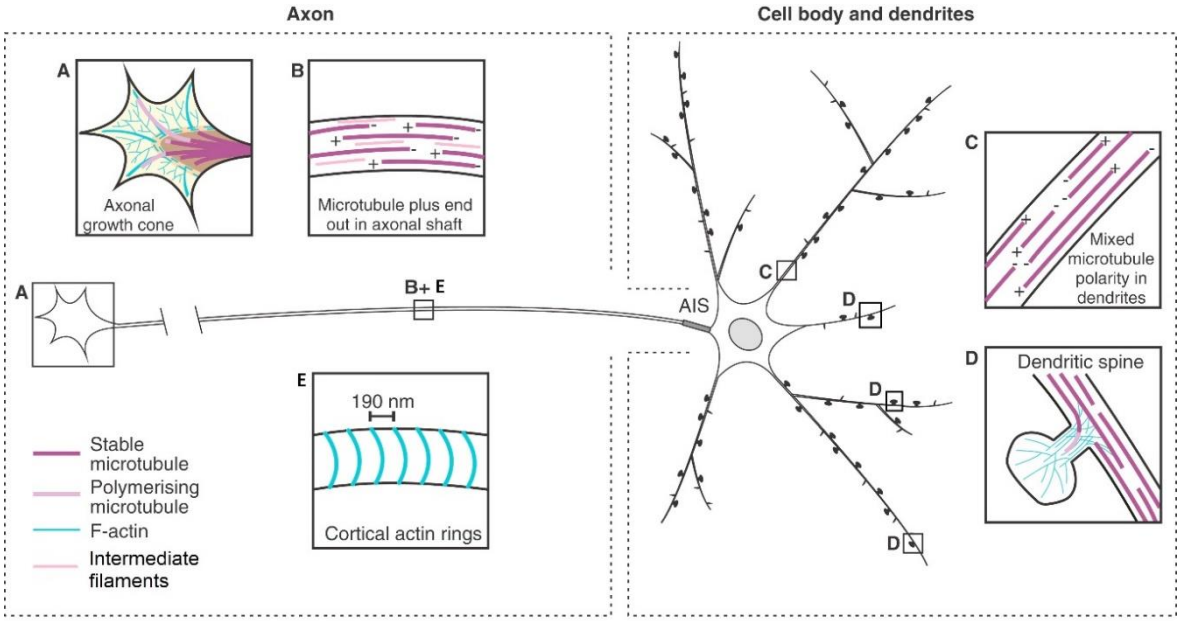

Figure 1 – Schematic representation of the cytoskeleton components in neurons. Cytoskeleton structures in axons and somato-dendritic compartments are depicted and boxes correspond to expanded views of the cytoskeleton organization in the respective areas. **(A)** In growing neurons, the axonal tip, named growth cone, is mainly shaped by actin arrangements (filipodia and lamellipodia), present in the peripheral and central regions, by a transition zone composed of actomyosin arcs, and by microtubules majorly present in the central domain, with few ones protruding along F-actin towards the cell edge. **(B, E)** The axonal shaft contains intermediate filaments, microtubules oriented with their plus end tips directed to the growth cone, and actin, present in different structures, namely the subcortical membrane periodic skeleton. **(C)** In the dendritic shaft microtubules have a mixed polarity. Actin and the subcortical membrane periodic skeleton are also present in dendrites. **(D)** Mature neurons develop actin-enriched membrane protrusions named dendritic spines. Adapted from (Coles and Bradke, 2015).

cytoskeleton components in the neuronal compartments will be addressed in the following sections with a focus on the actin cytoskeleton.

1.1 Intermediate filaments

IFs constitute the major family of cytoskeleton proteins, which is very heterogeneous, as different types of IF proteins are expressed (Yuan et al., 2012). In neurons there are 5 types of IFs expressed: type IV neurofilament (NF) triplet proteins (NF-light (NF-L), NF-medium (NF-M) and NF-heavy (NF-H)), α-internexin, and type III peripherin (Weber and Geisler, 1982; Troy et al., 1990; Fliegner et al., 1994). This cytoskeleton component promotes mostly tensile strength and stability, and it is not involved in polarity and cell motility. Structurally, all IF protein subunits are composed by a globular N-terminal head, a central alpha-helical rod region, and a variable C-terminal tail domain (Yuan et al., 2012). IF assembly initiates with the parallel formation of coiled-coil dimers of IF subunits, which occurs through their rod domain. Afterwards, two dimers associate in an antiparallel manner creating a tetramer. The arrangement of 8 tetramers constitutes a unit-length filament (ULF) with approximately 55 nm. The axial aggregation of ULFs promotes filament elongation and the subsequent radial compaction prompts the filaments packing resulting in the final 10nm (diameter) filament. The filaments core is comprised by the rod domains while the C-terminal tails constitute the flexible extensions that bind both the filaments to each other and the filaments to the other cell components, including other cytoskeleton constituents (Hisanaga and Hirokawa, 1988; Herrmann et al., 1999; Uchida et al., 2013). NFs assembly is mostly regulated by phosphorylation (Nixon and Sihag, 1991). In fact, NFs are the most phosphorylated proteins in the brain tissue further indicating the importance of this modification for the regulation and function of the NFs (Yuan et al., 2017). The NFs phosphorylation status comprises not only a signal for their assembly and organization but also determines their neuronal localization, which is predominant in axons and sparse in dendrites (Figure 1B) (Nixon and Lewis, 1986; Burton and Wentz, 1992).

1.2 Microtubules

MTs are tubular structures composed of parallel bundles generated by the polymerization of dimers of α- and β-tubulin. These polymers run along the axons and dendrites contributing to the neuronal structure and functioning, being the navigation pathways for axonal transport (Prokop, 2013). Tubulin is a superfamily of proteins and the α/β dimers of neuronal MTs are composed by the neuronal-specific isoform βIII-tubulin. Both α- and β-tubulin monomers bind GTP promoting polymerization; however, shortly after polymerization, β-tubulin-bound GTP is hydrolyzed to GDP weakening the affinity of tubulin

for the polymer (Mitchison and Kirschner, 1984). This results in the characteristic process of MTs known as dynamic instability, which promotes MT growth or shrinkage depending on the relative concentrations of GTP-tubulin (Mitchison and Kirschner, 1984). MTs are polymerized structures with fast-growing plus end and a slow-growing minus end which are particularly evident in the treadmilling process (Walker et al., 1988). Specifically, MT treadmilling is a dynamic process in which tubulin is simultaneously polymerized at the plus end (filament growing) and removed from the minus end (filament shrinking) (Walker et al., 1988). This polarity is an important characteristic of MTs, contributing for the guided axonal transport carried out by the motor proteins kinesins and dyneins (Hirokawa et al., 2010). Importantly, neurons are highly polarized cells and MTs play a crucial role in determining cell polarity as they constitute the fundamental support for axons and dendrites. While in axons MTs are oriented with their plus ends towards the growth cone and the minus end towards the soma, in dendrites MTs present a mixed orientation (Figure 1B, C) (Burton and Paige, 1981; Heidemann et al., 1981; Baas et al., 1988). MTs are regulated by a group of proteins known as microtubule-associated proteins (MAPs), which impact on MT structure, stability, dynamics and its interaction with other cellular components (Takemura et al., 1992). The association of different MAPs with MTs is also responsible for the distinct organization of this cytoskeleton component in axons and dendrites. For instance, the specific MAP present in axons is tau while in dendrites is MAP-2 (Matus, 1990). Additionally, MT stability is also regulated by several post-translational modifications (PTMs) in which the most studied ones comprise tubulin tyrosination/detyrosination (Gundersen et al., 1984), tubulin acetylation (Black and Keyser, 1987) and tubulin glutamylation (Edde et al., 1990).

1.3 Actin filaments

Actin filaments (F-actin) are formed by the association of globular actin (G-actin) to a growing polymer (Mitchison and Cramer, 1996). G-actin is linked to a nucleotide that varies between ADP and ATP (Atkinson et al., 2004). Upon actin assembly, ATP is rapidly hydrolyzed into ADP·Pi, which gradually releases the Pi (inorganic phosphate) generating ADP-actin. F-actin is a polarized filament with different rates of monomer assembly and disassembly with the plus end being fast growing and the minus end of slow growing. Actin monomers polymerization/depolymerization from the actin filament constitute the fundamental process driving actin dynamics (Carlier, 1998). These filaments can build into higher order structures in different regions of the neuron, a process highly dependent on the action of actin-binding proteins (ABPs).

1.3.1 Actin-binding proteins

ABPs constitute a group of proteins essential for actin cytoskeleton regulation. There is a considerable number of ABPs that controls the assembly and disassembly of F-actin regulating the dynamics of the actin filaments: (i) nucleation factors, as formins and Arp2/3, that promote the assembly of G-actin into filaments promoting polymerization and branched networks (Bugyi et al., 2006; Korobova and Svitkina, 2008); (ii) actin monomer binding proteins, such as profilin, which are fundamental for actin polymerization as they promote ADP to ATP nucleotide exchange on actin, providing new G-actin subunits to the filament (Mockrin and Korn, 1980); (iii) proteins that bundle, like fascin, or crosslink, such as α-actinins and filamins, the actin filaments (Tseng et al., 2004); (iv) actin filament stabilizing proteins, such as tropomyosin, which is able to regulate actin dynamics through binding along F-actin and interaction with other ABPs, or by direct contact of F-actin with different tropomyosin isoforms (Gunning et al., 2008; Schevzov et al., 2012), or drebrin, which interaction with F-actin results in the stabilization of the filament and inhibits the binding of several other ABPs (Asada et al., 1994); (v) spectrins that are localized at subcortical regions linking the actin cytoskeletal meshwork to membrane receptors (Burridge et al., 1982); (vi) capping proteins, including adducin and cyclase-associated protein 1 (CAP1), which bind to actin filaments blocking their growth (Matsuoka et al., 2000) and (vii) severing proteins, namely actin depolymerizing factor (ADF)/cofilin and gelsolin, which control the rate of actin depolymerization (Weeds et al., 1991; Andrianantoandro and Pollard, 2006).

The activity and localization of the ABPs is under the control of several molecular pathways and signaling cascades. Rho GTPases are at the center of these signaling pathways and, once activated, Rho GTPases signal a myriad of effectors including protein kinases and ABPs (Lee and Dominguez, 2010). The three most extensive studied Rho GTPases that drive cytoskeleton remodeling are RhoA, Rac1, and Cdc42 (Negishi and Katoh, 2002). The activation status of Rho proteins is regulated by its binding to either guanosine triphosphate (GTP, activation) or guanosine diphosphate (GDP, inactivation). Local guanine-nucleotide exchange factors (GEFs) or GTPase-activating proteins (GAPs) provide for the activation or inactivation of Rho GTPase proteins, respectively (Figure 2) (Luo, 2000). Rho GTPases signaling pathways induce different actin cytoskeleton rearrangements, with the activation of Rac1 preferentially leading to lamellipodia formation, the activation of Cdc42 resulting in filopodia and RhoA inducing stress fibers (Figure 2) (Nobes and Hall, 1995). Within the variety of proteins regulated by Rho GTPases are included the ABPs cofilin, profilin and Arp2/3.

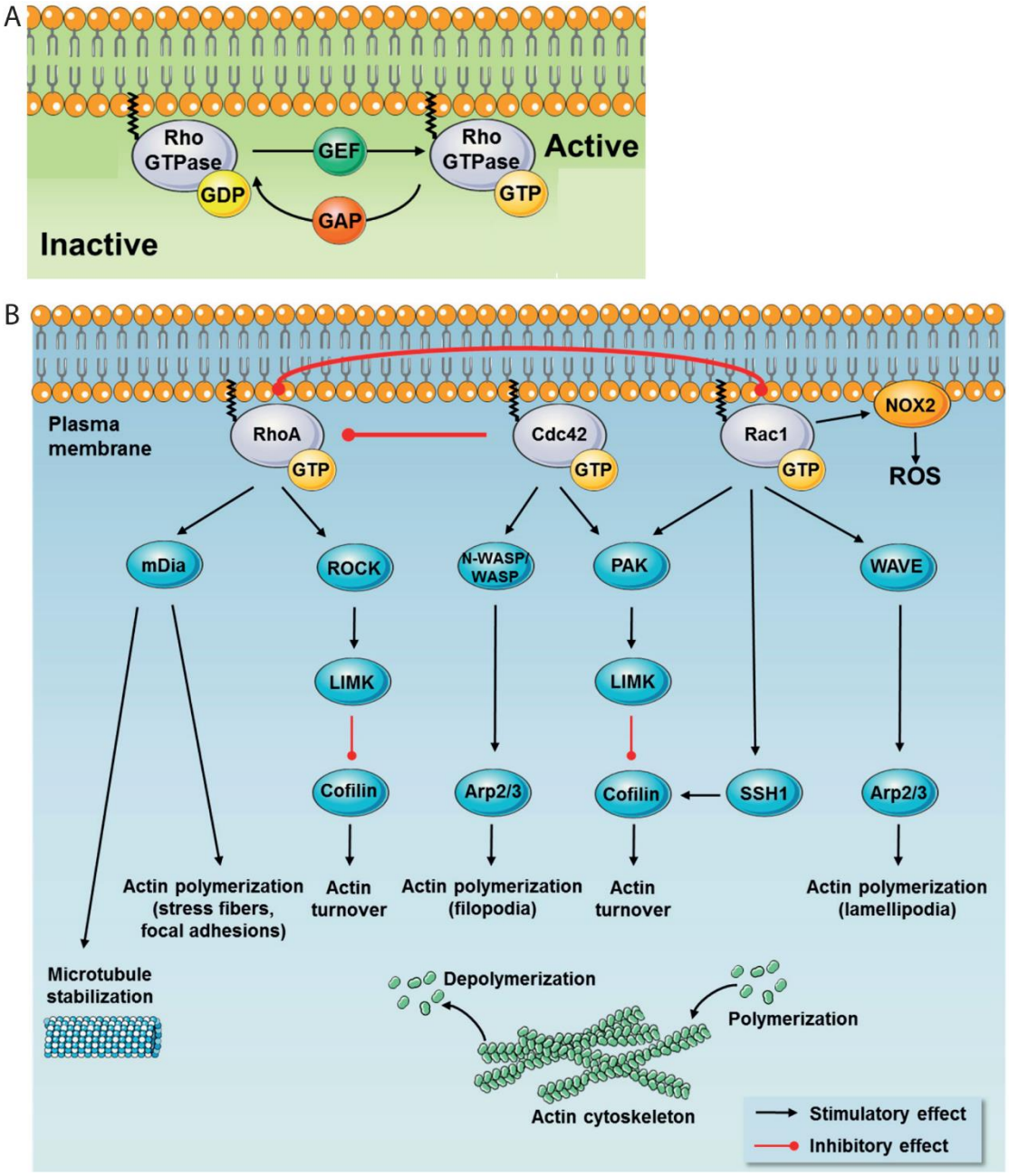

Figure 2 - Regulation and downstream effectors of Rho GTPases. (A) Activation of Rho GTPases requires the action of GEFs, which promote GDP to GTP nucleotide exchange, and inactivation requires GAPs, that stimulate GTP hydrolysis into GDP. **(B)** Downstream effector proteins of RhoA, Cdc42 and Rac1 including several proteins regulating actin cytoskeleton dynamics. Adapted from (Moller et al., 2019).

1.3.2 Actin polymerization and remodeling

Actin polymerization and remodeling is an orchestrated process which occurs in several stages (Figure 3). Firstly, in the nucleation phase, monomers of ATP-bound G-actin associate forming the nucleus. Next, the addition of actin monomers at both ends of the nucleus contributes for filament growth (elongation phase). In this phase, the barded end (plus end) grows much faster than the pointed end (minus end) (Woodrum et al., 1975;

Pollard and Mooseker, 1981). With this process, the pool of free G-actin decreases until reaching a state where there is a balance between F-actin and G-actin. However, the filament is not static but rather a dynamic structure undergoing constant actin turnover, a process known as actin treadmilling (Neuhaus et al., 1983). This process is highly controlled by different ABPs, but there are two specific ABPs that are crucial for the process to occur, namely profilin and cofilin (Figure 3). Profilin binds to G-actin coupled to ADP and promotes the exchange of ADP to ATP (Vinson et al., 1998). Then, the complex profilin-ATP-G-actin is attached to the filament barbed end, where profilin provides the new actin subunit to the filament and dissociates itself from the filament (Pring et al., 1992). This results in increased actin polymerization. Cofilin has high affinity for ADP-actin-enriched regions of the actin filament thus binding specifically to the pointed end which contains the initial added actin subunits (Van Troys et al., 2008). Cofilin binding to two actin monomers induces filament rotation and promotes the destabilization and breakage of the filament into smaller fragments (severing) or monomer dissociation from the filament, resulting in increased actin disassembly (Carlier et al., 1997). Interestingly, the ADP-G-actin subunits released in this process can be captured by profilin, reinitiating the polymerization cycle. Taking this into consideration, the synergy between cofilin and profilin activity is crucial to correctly regulate actin dynamics.

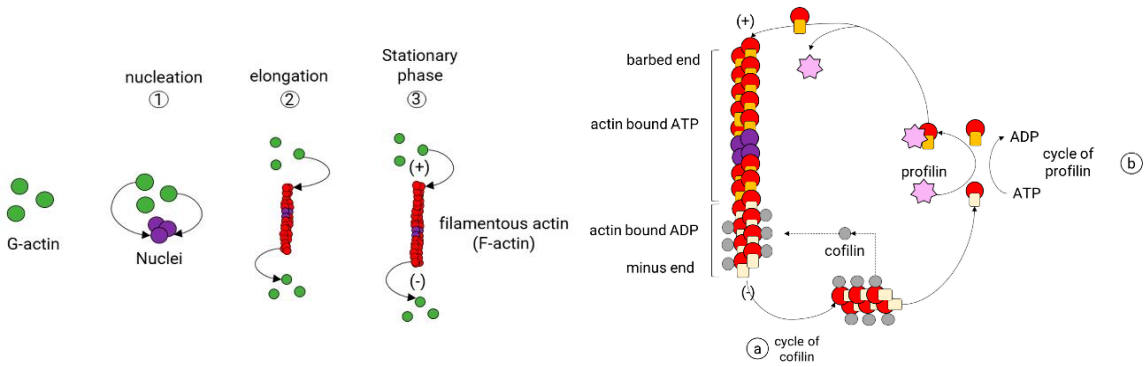

Figure 3 - Schematic representation of actin polymerization and treadmilling. ATP-G-actin monomers (green) aggregate forming the nucleus (nucleation phase, 1), then G-actin monomers are added to both ends of the nucleus (elongation phase, 2), and lastly actin filament reaches a stationary phase (3). The filament is not static and is constantly undergoing assembly/disassembly – treadmilling. Cofilin (a) binds ADP-actin at the minus end of actin filaments promoting fragmentation. Profilin (b) binds ADP-G-actin and catalyzes the conversion of ADP to GTP on actin. Profilin-ATP-G-actin complex binds to the barbed end of F-actin promoting polymerization. Adapted from (Munoz-Lasso et al., 2020).

1.3.3 Actin architecture in different neuronal compartments

In neurons, actin acquires several structural arrangements that are differentially distributed in the cell (Figure 1 and 4). Lamellipodia is an actin structure characterized by a branched network of short actin filaments while filopodia is composed of long parallel bundles of actin filaments (Neukirchen and Bradke, 2011). Lamellipodia and filopodia are actin arrangements enriched in the more dynamic neuronal structures including the growth cone, a structure assembled during axonal growth (Figure 4B). In the growth cones, besides the peripheral domain enriched in filopodia and lamellipodia, there is, in the transition zone, an actomyosin contractile structure named actin arcs which are perpendicular to F-actin and are suggested to interact with MTs and allow them to invade the growth cone (Schaefer et al., 2002). Actin dynamics is a fundamental process that provides the required forces for the protrusion of the growth cone.

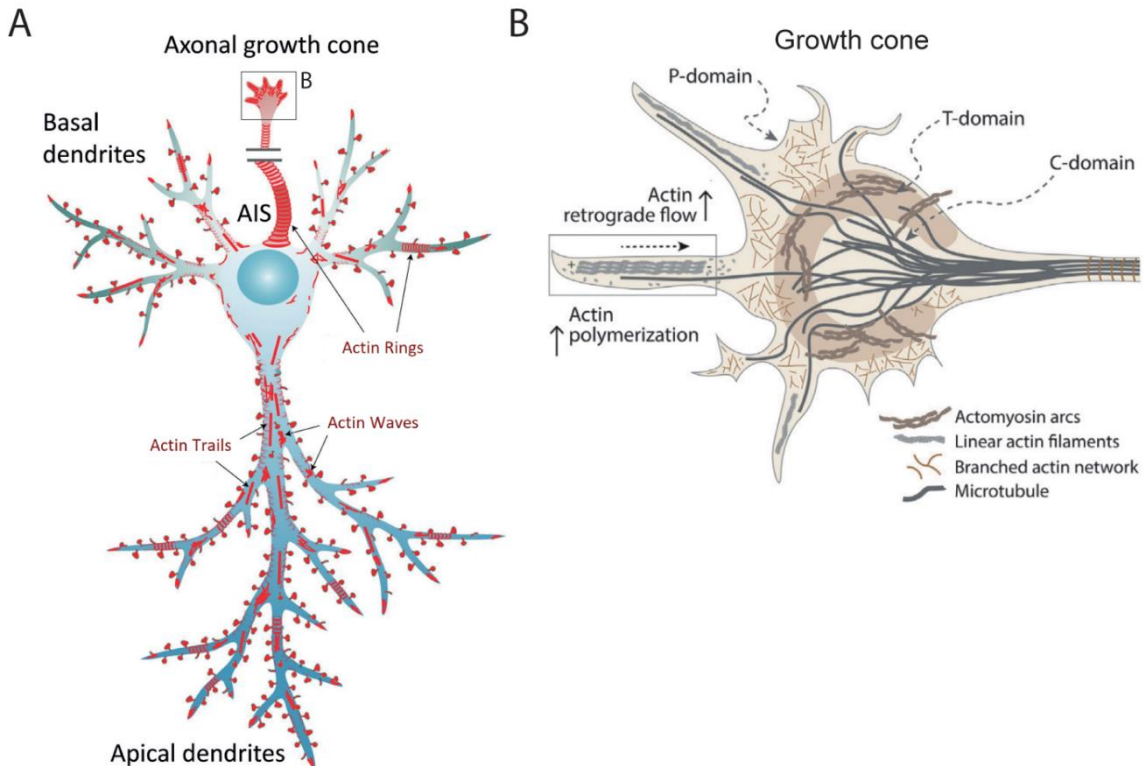

Figure 4 – Overview of the actin cytoskeleton organization in neurons. **(A)** Depiction of F-actin enriched structures (red) in the different neuronal compartments, including actin rings, actin waves and actin trails in neurites, and actin enrichment in growth cones. **(B)** Actin structures in growth cones comprising actin filaments (filopodia), branched actin (lamellipodia) in the peripheral domains and actomyosin arcs in the central domain. Adapted from (Konietzny et al., 2017) and (Leite et al., 2020).

With the development of superresolution microscopy techniques, new actin structures have been described (Figure 5). Firstly, a component of the neuronal subcortical cytoskeleton was described, the membrane periodic skeleton (MPS) (Xu et al., 2013). This structure, also named as “actin rings”, is present along the axon and dendrites and is

composed by rings of short actin filaments capped by adducin distanced by approximately 190 nm by spectrin (Xu et al., 2013). Recently, non-muscle myosin II was shown to be part of the MPS structure and proposed to regulate the axonal actin ring contraction and expansion (Costa et al., 2020). While actin rings are considered to give mechanical support to neurons, there is a dynamic pool of actin in the structure of the axon, termed actin trails, which has been suggested to be composed of actin "hotspots" and to provide for a flexible actin cytoskeleton network in the axon (Ganguly et al., 2015). Additionally, actin waves have also been described as structures present in growing neurons occurring in the shaft and migrating to the edge of the neurites and protruding into the plasma membrane (Flynn et al., 2009). These structures are believed to be similar to growth cones as G-actin is added at the leading edge of the actin filament and is disassembled at the base.

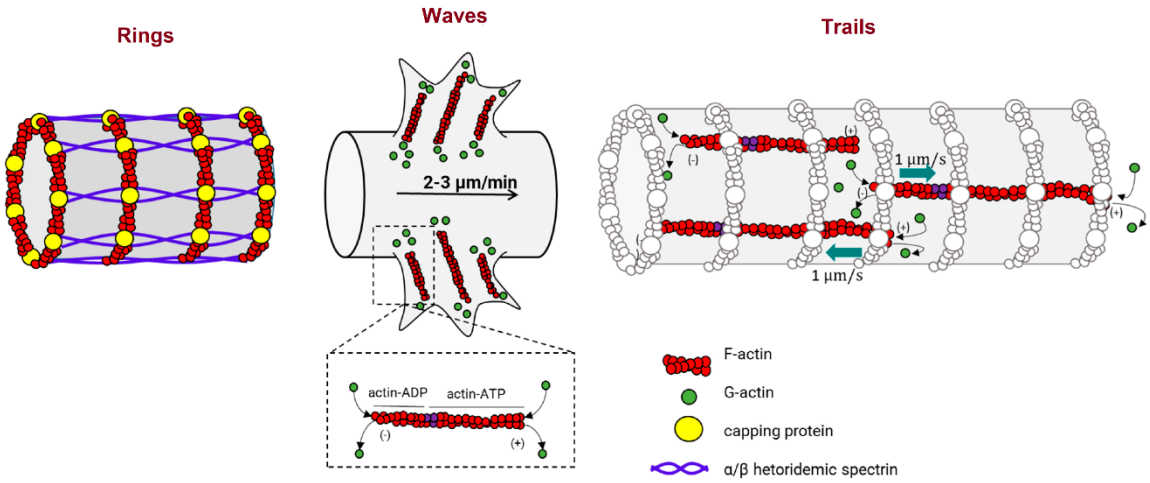

Figure 5 - Recently described actin structures. Schematic representation of the three actin structures described more recently, namely actin rings, present in dendrites and axons, actin waves and actin trails, present in axons. Adapted from (Munoz-Lasso et al., 2020).

1.3.3.1 Dendritic spines

Synapses constitute the functional units in neurons and are composed by the presynapse, present at the axon terminal, and the counterpart postsynapse, which is along the dendrites and typically denominated by dendritic spines. Both synaptic structures are enriched on actin which determines a fundamental role of this cytoskeleton component for synaptic transmission (Waites and Garner, 2011). In fact, in mature neurons the most striking actin-enriched structure in dendrites are the dendritic spines (Basu and Lamprecht, 2018). In fact, the concentration of actin in dendritic spines is around 18 times higher than the one in the dendritic shaft corroborating the importance of the actin cytoskeleton in this structure (Capani et al., 2001). Spine maturation is characterized by a process where a filopodia-like protrusion emerges from the dendritic shaft, which undergoes constant actin

polymerization and depolymerization, reaching a mature stage when the spine presents the characteristic head and small neck (Bourne and Harris, 2008). Mature spines are characterized by a mushroom shape in which the head contains a branched actin network, connected to the dendritic shaft by a narrow neck composed of parallel actin filaments (Harris et al., 1992). Spines can vary in size and shape originating other spine morphologies such as stubby spines, which have a defined head without a visible neck, thin spines, which have a small head with a long neck, and filopodia-like spines, which present a defined neck without a head (Bourne and Harris, 2008). The distinct spine morphology implies an underlying cytoskeleton structure, and actin dynamics constitutes the major contributor in the process of spine shaping. Accordingly, one characteristic of dendritic spines is their structural modification according to synaptic activity. During long term potentiation (LTP) there is a shift in actin dynamics towards F-actin polymerization resulting in spine enlargement (Bosch et al., 2014), while during long term depression (LTD) occurs the opposite, a shift in actin dynamics towards depolymerization (more G-actin), culminating in spine shrinkage/elimination (Okamoto et al., 2004) (Figure 6). Considering the importance of actin polymerization and depolymerization cycles for spine plasticity and the role of ADF/cofilin on this process, several reports have evidenced that ADF/cofilin is a central regulator of dendritic spine morphogenesis and dynamics (Noguchi et al., 2016). In fact, ADF/cofilin contributes not only to the maintenance of a rapid actin treadmilling but also to ensure a proper actin filament severing in the spine neck and head, preventing the abnormal plasma membrane protrusions in dendritic spines (Hotulainen et al., 2009).

1.3.4 Cofilin-1: a critical ABP

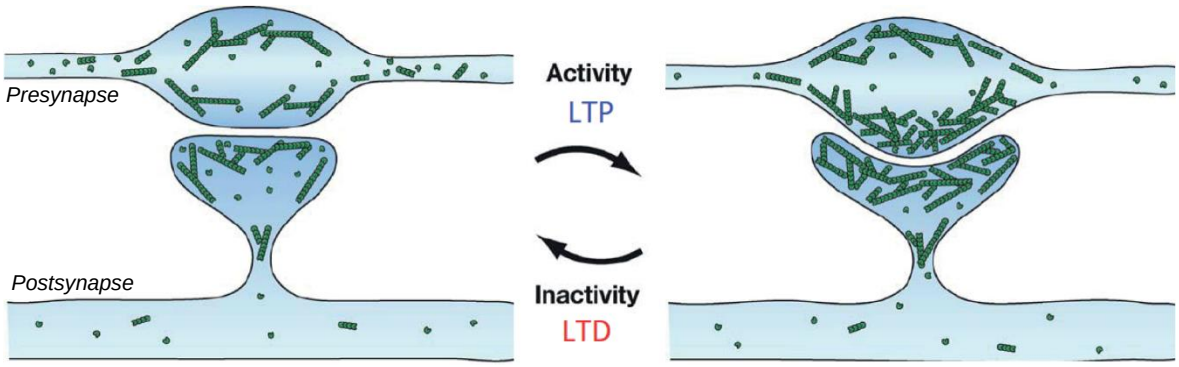

Figure 6 – Representation of actin in synapses. Activity-dependent changes in actin (green) at the pre and postsynapse are represented. Under stimulation (LTP) F-actin increases in both sides of the synapse and the postsynaptic side (dendritic spine) remodels and expand the contact area with the presynapse. Inactivity (LTD) induces actin dynamics towards G-actin and eventual regression of spines. Adapted from (Dillon and Goda, 2005).

The ADF/cofilin proteins are present in all eukaryotes, and mammals express three different forms of these proteins: ADF, cofilin-1 and cofilin-2 (majorly present in muscle) (Bamburg, 1999). Among the three proteins, cofilin-1 is the most ubiquitous and in neurons

is expressed five to ten times higher than ADF (Minamide et al., 2000), and as such will be the focus hereafter and referred as cofilin. Cofilin has a strong affinity for filamentous ADP-actin and a weak affinity for ATP-actin subunits, and its function on actin dynamics is tightly associated with its concentration in relation to actin and to other ABPs (Van Troys et al., 2008). At low cofilin/actin ratios, cofilin acts as an actin severing protein, and at high cofilin/actin ratios, cofilin acts as an actin stabilizer and nucleation protein (Andrianantoandro and Pollard, 2006).

Cofilin activity and actin-binding function are regulated by different processes. The interaction of cofilin with other proteins impacts on its activity towards actin, and amongst these proteins are included: i) tropomyosins, which are competitors of cofilin for F-actin binding, contributing to the stabilization of the filament (Bryce et al., 2003); ii) cortactin, an ABP of the cell cortex that competes with cofilin for binding to actin subunits (Bryce et al., 2005); iii) CAP1, that alone do not impact directly on actin stability but enhance cofilin-stimulated filament turnover, through displacement of cofilin from ADP-actin (Moriyama and Yahara, 2002; Balcer et al., 2003); iv) actin-interacting protein (Aip1), which improves the severing and depolymerizing activity of cofilin through binding along cofilin-saturated F-actin (Okreglak and Drubin, 2010); and v) coronin, which effect on cofilin-actin interaction depends on the nucleotide that is bound to actin. In regions enriched with ADP-actin, coronin potentiates cofilin severing while in regions of high ATP-actin coronin binds competitively diminishing the binding of cofilin to actin (de Hostos, 1999; Cai et al., 2007; Gandhi et al., 2009).

Besides protein-protein interactions, cofilin activity is mainly regulated by phosphorylation at its serine 3 (Ser3) residue which inhibits its binding to actin (Figure 7) (Morgan et al., 1993; Moriyama et al., 1996). Phosphorylation of Ser3 on cofilin can be accomplished by two families of kinases, LIM kinase (LIMK) and TES kinase (Arber et al., 1998; Toshima et al., 2001). LIMK is regulated by proteins such as Rho kinase (ROCK) and p21-activated kinase (Pak1), which are upstream regulated by Rho GTPases (Edwards et al., 1999). The regulation of TES kinases in not yet well understood. Cofilin dephosphorylation may be regulated by different phosphatases including chronophin (CIN) and slingshot homologue-1 (SSH1), leading to cofilin activation (Niwa et al., 2002; Gohla et al., 2005). Cofilin activation through dephosphorylation by SSH1 requires the binding of the enzyme to F-actin, providing an additional level of regulation that restricts cofilin activation at sites with relatively high levels of F-actin (Nagata-Ohashi et al., 2004). Moreover, SSH1 is inhibited by phosphorylation at Ser978 by protein kinase D (Eiseler et al., 2009). Further regulation is provided by 14-3-3 proteins that sequester phosphorylated SSH1 preventing its translocation to sites where it can be activated (Nagata-Ohashi et al., 2004). Calcineurin, which activity is calcium dependent, is responsible for the dephosphorylation and

consequent activation of SSH1 (Wang et al., 2005). Interestingly, SSH1 was described to interact and dephosphorylate (inactivate) LIMK, thereby adding a further degree of regulation of cofilin activation (Soosairajah et al., 2005).

There are additional mechanisms for the regulation of cofilin activity which are independent of phosphorylation, namely the interaction of cofilin with membrane phosphatidylinositol 4,5-bisphosphate (PIP_2), which inactivates the ABP activity (Yonezawa et al., 1990). Another relevant level of regulation is the intracellular pH that affects PIP_2 density at the membranes, which in turn influences cofilin release and activation (Frantz et al., 2008). Cofilin-dependent actin severing and depolymerization are increased with higher intracellular pH levels (pH=8 when compared with pH=6.5) (Yeoh et al., 2002). Additionally, an increased pH results in the release of cofilin from cortactin inhibition potentiating cofilin activation (Bryce et al., 2005). Other mechanism of cofilin regulation is oxidation (Bernstein and Bamburg, 2010). Reactive oxygen species (ROS) play an important role as signaling molecules in different processes. ROS are capable to oxidize 14-3-3ζ promoting the SSH1 release from its inhibition, which becomes available to dephosphorylate (activate) cofilin (Kim et al., 2009). Additionally, oxidative stress also affects mitochondria dysfunction, and once the mitochondrial membrane potential drops, is verified a rapid activation of cofilin resulting in cofilin-saturated F-actin (Minamide et al., 2000; Bamburg and Wiggan, 2002). The direct oxidation of cofilin also modulates its activity. Recently, it was shown that cofilin can be inhibited through oxidation of two cysteines (Cys139 ad Cys147) by endogenous peroxide (Cameron et al., 2015). However, it was previously proposed that exogenous peroxide enhances cofilin activity (Taulet et al., 2012), so further investigation will be necessary to clarify these data.

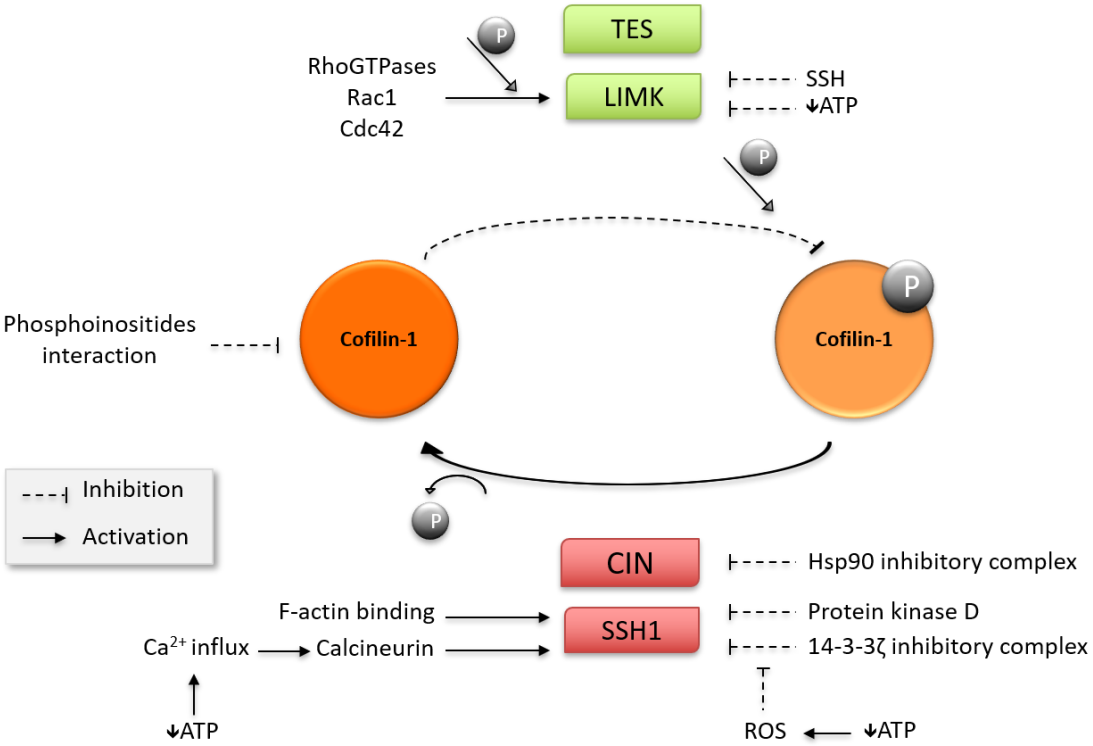

Figure 7 - Mechanisms of cofilin regulation. Representation of cofilin-1 regulation by the different molecular pathways reported. The dashed and solid lines represent, respectively, inhibitory and activation mechanisms. The detailed description is found along the text. Adapted from (Schonhofen et al., 2014).

2 Cytoskeletal abnormalities in neurodegeneration

Considering the high density of cytoskeleton proteins as well as their highly regulated function in neurons, as evidenced in the previous section, it is conceivable that cytoskeleton damage has been implicated in several neurodegenerative disorders (Eira et al., 2016). In neurodegenerative disorders, the destabilization of the cytoskeleton architecture and dynamics might arise from different causes including mutations in cytoskeleton genes, mutations in genes encoding proteins that regulate the cytoskeleton or protein dysregulation that results in cytoskeleton damage. From the various cytoskeleton dysfunctions, alterations in MT stability are a main causative agent in several neurodegenerative disorders and include: i) variations in the levels of tubulin PTMs with a consequent impact on axonal transport (Dompierre et al., 2007; d'Ydewalle et al., 2011; Zhang et al., 2014; Qu et al., 2017; Magiera et al., 2018), and ii) dysregulation of MT associated proteins, such as the key example of tau hyperphosphorylation. This modification promotes tau detachment from MTs, what either impacts on axonal transport (Alonso et al., 1997) or leads to recruitment of MT severing enzymes causing axon degeneration (Qiang et al., 2006). Abnormalities related with IFs can result in axonal transport defects and reduction of the axon caliber. Mutations in NF genes are causative of autosomal Charcot-Marie-Tooth (CMT) disease, a sensory-motor neuropathy affecting the peripheral nervous system (Perez-Olle et al., 2005). Disruption and aggregation of NFs can lead to motor neuron degeneration contributing to neuronal loss and disease progression in CMT (Zhai et al., 2007). Concerning the actin cytoskeleton, mutations on actin or its regulatory proteins (eg. ABPs) is sufficient to trigger actin cytoskeleton damage in neurons. Actin cytoskeleton destabilization might have an impact in: (i) neurite outgrowth during development, where the actin cytoskeleton plays a crucial role in the dynamicity of the growth cone; (ii) abnormal formation or deficient maintenance of dendritic spines, affecting synaptic function and (iii) formation of pathological inclusions composed of cofilin-saturated F-actin, namely cofilin-actin rods (Munoz-Lasso et al., 2020). These actin cytoskeleton abnormalities in neurodegeneration will be further dissected in the following section.

2.1 Actin cytoskeleton dysfunction in neurodegenerative diseases

The actin cytoskeleton is involved in several neuronal functions and there is a plethora of proteins that directly or indirectly regulate the actin dynamics process. When one of these proteins is mutated or dysregulated, the consequence might be a defective actin cytoskeleton organization and consequent development of disease, as occurs in several neurodegenerative disorders.

Mutations in actin binding proteins

Focusing on diseases caused by mutations in cytoskeleton-related genes, the first description of mutations in the gene encoding for the ABP profilin-1 (*PFN1*) as a causing agent of familial Amyotrophic Lateral Sclerosis (ALS), a disorder characterized by the progressive loss of motor neurons in brain and spinal cord, confirmed the direct link between actin cytoskeleton dysregulation and neurodegeneration (Wu et al., 2012). Several mutations in *PFN1* were detected in ALS patients and the expression of these mutants in cultured neurons resulted in decreased actin-binding affinity in profilin-1, causing axon outgrowth inhibition (Wu et al., 2012). Additionally, some of the described *PFN1* mutations were found to cause detergent-resistant protein aggregates formation, while others interfere with phosphorylation sites in profilin-1 inhibiting its interaction with other molecular partners (Ingre et al., 2013; Smith et al., 2015). Mutations in *SPTBN2* gene encoding for the ABP β-spectrin were shown to be associated with the neurodegenerative disorder Spinocerebellar ataxia type 5 (SCA5), a disease characterized by cerebellar symptoms (ataxia, dysarthria, tremor) and eye movement abnormalities (Elsayed et al., 2014). One of the described mutations was L253P, localized in the actin-binding domain of β-spectrin, that induces a higher affinity for actin which leads to decreased actin-spectrin dynamics resulting in impaired neurite outgrowth and compromised remodeling of dendrites and dendritic spines (Avery et al., 2016; Avery et al., 2017). Patients with CMT disease also present mutations in an ABP gene, namely *INF2,* encoding for the inverted formin-2 (Boyer et al., 2011). Mutations in this formin cause actin cytoskeleton dysregulation, interfering with myelination, resulting in the disturbance of Schwann cells polarization and consequent axon loss (Mathis et al., 2014).

Disruption of major regulators of actin dynamics

In addition to ABPs mutations, the dysregulation of signaling pathways controlling actin dynamics constitutes a major cause of actin alterations and has been in the center of intensive research. As mentioned above, the Rho GTPases pathway is a central signaling cascade controlling actin dynamics and its disruption has been associated with the development of several neurodegenerative disorders. In CMT, mutations in frabin/FDG4, a GEF specific to Cdc42, induced peripheral nerve demyelination (Delague et al., 2007; Stendel et al., 2007). In Huntington's disease (HD), where neuronal intranuclear accumulation of aggregated huntingtin results in motor, psychiatric, and cognitive symptoms, several members of the Rho GTPases pathway were described to be modifiers of the huntingtin-induced toxicity phenotype, which raises the hypothesis that huntingtin pathology might interfere with cytoskeleton-related processes such as cell attachment, membrane dynamics and motility (Tourette et al., 2014). Additionally, the RhoA signaling

pathway has been implicated in Parkinson's disease (PD), a neurodegenerative disorder mostly characterized by αSynuclein (αSyn) aggregation and motor impairment. In this respect, a PD mouse model treated with a ROCK inhibitor (fasudil) presented a significant attenuation of dopaminergic cell loss in substantia nigra (Tonges et al., 2012). Concerning Alzheimer's disease (AD), a neurodegenerative disorder characterized by accumulation extracellular amyloid plaques (composed by amyloid-β (Aβ)) and intraneuronal neurofibrillary tangles (composed by tau), resulting in memory impairment, several evidences demonstrated the involvement of Rho GTPases in the disease. Increased levels of RhoA were observed in the cerebral cortex of an AD mouse model (Petratos et al., 2008), and hippocampal neurons treated with Aβ showed altered levels of Cdc42 and Rac1 with consequences for the actin cytoskeleton organization, including dysregulated actin polymerization (Mendoza-Naranjo et al., 2007).

Dysregulation of actin binding proteins

The dysregulation of several ABPs, independently of genetic mutations, has also been reported in numerous neurodegenerative disorders. Drebrin plays crucial roles during axon growth, neuronal migration and synapse formation. Specifically, drebrin interaction with the actin cytoskeleton is essential for the maintenance of dendritic spine density and morphology (Harigaya et al., 1996). Drebrin was found to be decreased in AD brains suggesting a link between actin dysregulation and synaptic dysfunction in AD (Harigaya et al., 1996). In fact, synaptic dysfunction in AD was described to occur through Aβ accumulation and consequent reduction of Pak1 activity, which led not only to decreased drebrin levels but also to cofilin activation, culminating in the dysregulation of actin dynamics (Kojima and Shirao, 2007). Drebrin and cofilin competitively bind to actin filaments and while cofilin promotes actin severing, drebrin stabilizes F-actin and inhibits cofilin-mediated actin severing (Grintsevich and Reisler, 2014). Thus, the correct control of cofilin and drebrin activity is crucial to maintain the proper actin architecture and dynamics in dendritic spines.

2.1.1 Cofilin involvement in neurodegeneration

Within the several ABPs, cofilin dysfunction has been attracting higher attention due to its increasing association with several unrelated neurodegenerative disorders. The consequences of cofilin dysregulation usually comprises neurotoxicity and neuroinflammation. When the outcome of cofilin dysregulation results in its hyperactivation, a major consequence is the formation of neurodegeneration-related structures: the cofilin-actin rods, which being the major focus of this thesis will be detailed in this section. Hirano bodies are additional neuropathologic structures containing cofilin that accumulate in AD

(Schmidt et al., 1989). Unlike cofilin-actin rods, these are not strictly composed by cofilin and actin but are enriched in several ABPs being cofilin one of the major constituents (Izumiyama et al., 1991).

2.1.1.1 Cofilin-actin rods assembly and structure

Cofilin-actin rods are structures formed upon cofilin hyperactivation, by dephosphorylation, and association with F-actin, leading to the formation of cofilin-actin fragments, which in the presence of ROS associate with each other creating bundles of cofilin-saturated actin filaments, the cofilin-actin rods (Figure 8) (Minamide et al., 2010). In neurons, the ratio between cofilin and actin is approximately 1:5 and this ratio changes to 1:1 prior to rod formation. Structurally, it was determined by electron microscopy that the average filament length within a rod structure is about 200 nm and the diameter of the individual filaments is approximately 9 nm (Minamide et al., 2010). Curiously, rods are not stained with phalloidin, a mushroom toxin with F-actin binding properties (McGough et al., 1997). This occurs due to the saturation of F-actin with cofilin that changes the filament twist disabling the simultaneous binding of cofilin and phalloidin to actin.

Cofilin-actin rods appear as single structures or as linear arrays, mainly within neurites, and in some cases, they can be large enough to occlude the neurite inhibiting axonal transport and blocking distal neurite function (Cichon et al., 2012). As an example of this effect, the impairment of mitochondrial movement has consequences on the energy supply at the synaptic structures, contributing to synapse loss (Cichon et al., 2012).

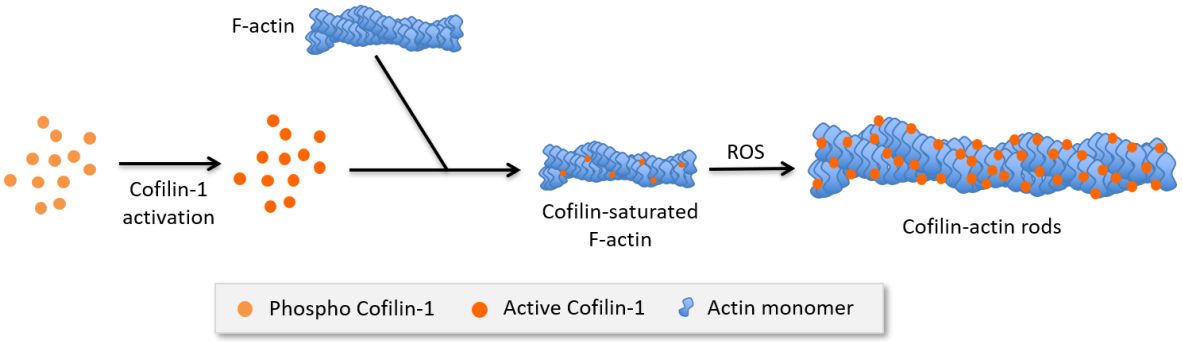

Figure 8 - Representation of cofilin-actin rods formation. High levels of activated cofilin bind to actin filaments forming short but stable cofilin-saturated actin fragments; the presence of ROS triggers an intermolecular disulfide bond between two cofilin molecules leading to the associated of several fragments resulting in cofilin-actin rod formation.

The essential factors for rod formation are high levels of active dephosphorylated cofilin, ADP-enriched actin, and an oxidative environment (Figure 8) (Minamide et al., 2000; Bernstein et al., 2012). The referred conditions are easily found in stressed neurons; however, the formation of rods may have a temporarily neuroprotective effect by allowing

cytoskeleton dynamics arrest in cells under stress (Figure 9) (Bernstein et al., 2006). Actin turnover requires high levels of ATP and the sequestration of cofilin in rods reduces that process and concomitant ATP hydrolysis, contributing to a slower decline in ATP levels and mitochondrial membrane potential under stress conditions (Bernstein et al., 2006). Activated cofilin is predominantly targeted to the mitochondria causing the release of cytochrome c that ultimately induces apoptosis (Figure 8) (Chua et al., 2003). Therefore, the formation of rods transiently inhibits the translocation of cofilin to the mitochondria, preventing temporarily neuronal death. Additionally, during rod formation a single intermolecular disulfide bond in cofilin is produced reducing the availability of the protein to form two intramolecular disulfide bonds that would target cofilin to the mitochondria (Klamt et al., 2009; Bernstein et al., 2012). Importantly, rods dissociate quickly following the relief of the stress stimuli. Since rods are temporarily reversible, they appear to promote neuronal viability even in the presence of synapse loss. However, persistent rods result in neuronal damage as will be later discussed.

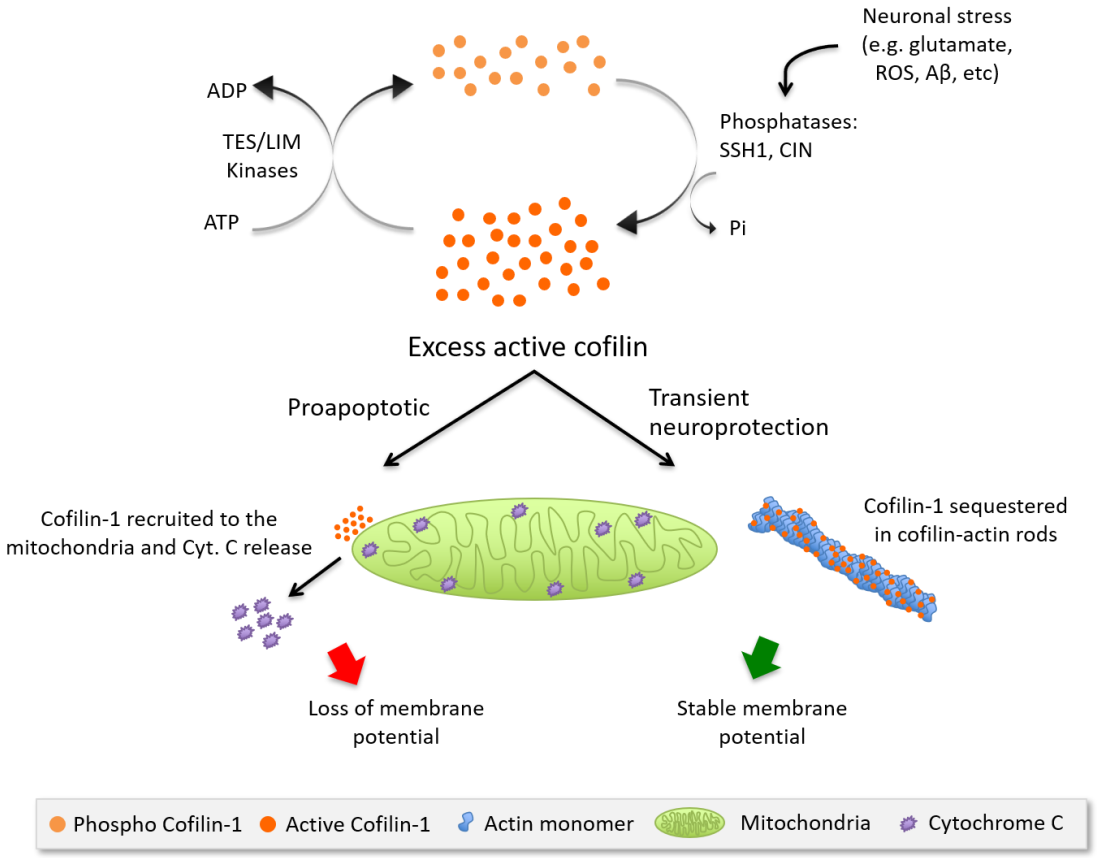

Figure 9 - Schematic representation of the neuroprotective effect of rods. Brief elucidation of beneficial effects of cofilin-actin rods formation in the delayed neuronal death through temporary inhibition of cytochrome c release. Adapted from (Maloney and Bamburg, 2007).

2.1.1.1 Signaling pathways involved in cofilin-actin rods formation

Several neurodegenerative stimuli able to cause rod formation have been described, including high levels of glutamate, mitochondrial inhibitors, nitric oxide, hydrogen peroxide, proinflammatory cytokines and Aβ (Figure 10) (Minamide et al., 2000; Maloney et al., 2005; Bernstein et al., 2006; Davis et al., 2009; Cichon et al., 2012; Walsh et al., 2014). Depending on the stress agent, different pathways involved in rod formation were reported. However, in general these pathways culminate in ROS production and dephosphorylation of cofilin by either activating phosphatases or inhibiting kinases (Figure 9). In response to excitotoxic levels of glutamate, AMPA receptor is activated resulting in ATP depletion and consequent release of CIN from Hsp90 inhibition (Brennan et al., 2009; Bernstein et al., 2012; Walsh et al., 2014). Once activated, CIN dephosphorylates cofilin which becomes active and with the ability to incorporate into cofilin-actin rods. Externally applied ATP induces rod formation through the activation of the P2X receptor and subsequent calcium influx (Homma et al., 2008). High levels of calcium activate the calmodulin/calcineurin pathway resulting in the dephosphorylation (activation) of SSH1, which is able to dephosphorylate (activate) cofilin (Figure 10). The formation of rods in response to peroxide is also related to SSH1 activation. In this case, the sulfhydryl oxidation of 14-3-3ζ results in the release of SSH1 that becomes available to activate cofilin (Kim et al., 2009).

Regarding the pathways activated by proinflammatory cytokines and Aβ, they are tightly dependent on the cellular prion protein (PrP^C) and the activity of NADPH oxidase (NOX) (Figure 10) (Walsh et al., 2014). PrP^C is a protein anchored to the membrane outer leaflet that is typically enriched in sphingolipid membrane signaling domains (Hirsch et al., 2014). The overexpression of EGFP-PrP^C results in the increment of rod formation, with the concomitant addition of Aβ or proinflammatory cytokines not resulting in a further increase in rod formation (Walsh et al., 2014). This proposes that high levels of PrP^C are sufficient to induce rods and that rod formation in response to the two stimuli is dependent on the presence of PrP^C. When NOX inhibitors were used either in Aβ or proinflammatory cytokines treated neurons or in PrP^C overexpressing neurons, rod formation was reduced in all cases, indicating that Aβ and proinflammatory cytokines utilize a PrP^C-NOX dependent pathway to induce cofilin-actin rods (Walsh et al., 2014). Once activated, NOX leads to ROS production which triggers cofilin oxidation required for the formation of an intermolecular disulfide bond between cofilin molecules and subsequent incorporation into rods (Bernstein et al., 2012).

An additional pathway that was demonstrated to be involved in cofilin-actin rod formation downstream of Aβ involves the scaffolding protein RanBP9 (Figure 10). This protein was found with increased expression in AD brains and mouse models of AD (Lakshmana et al., 2010; Woo et al., 2012). RanBP9 is capable of inducing cofilin activation and to favor Aβ production (Lakshmana et al., 2009; Woo et al., 2012). In one hand, Aβ oligomers bind to β1-integrin receptors in a PrP^C-NOX dependent manner enhancing ROS production. In the other hand, RanBP9 can also positively regulate SSH1 levels, leading to cofilin dephosphorylation (Woo et al., 2012; Walsh et al., 2014; Woo et al., 2015a; Woo et al., 2015b). Together, these pathways culminate in cofilin-actin rods formation. In support of a link between Aβ, RanBP9 and cofilin-actin rods in AD, a transgenic mouse model generated by crossing APPsw/PS1ΔE9 mice with RanBP9+/- mice presented reduced

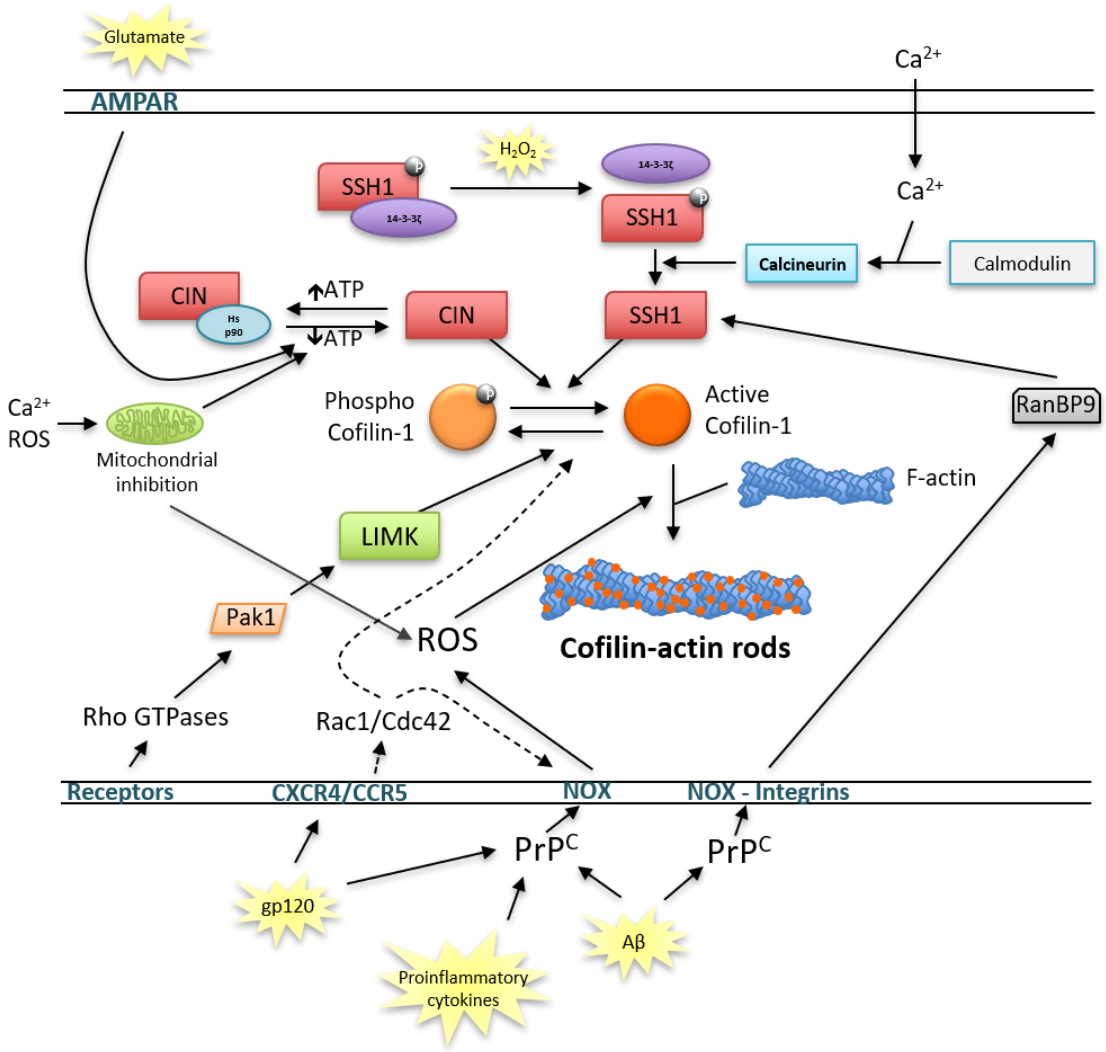

Figure 10 - Depiction of the stimuli and signaling pathways involved in neuronal rod formation. This simplified scheme elucidates the several stress inducers and the pathways mediating rod formation. An exhaustive description is present throughout the text. Filled arrows represent direct pathways and dashed arrows represent multi-step pathways. Adapted from (Bamburg and Bernstein, 2016) and (Smith et al., 2021).

levels of cofilin activation and rod formation, accompanied by an amelioration of the disease phenotype (Woo et al., 2012).

HIV-associated neurocognitive disorder (HAND) arise from the human immunodeficiency virus (HIV) infection in the brain inducing several neurotoxic effects which are considered to be the cause of synaptic degeneration and cognitive impairment in this disease. gp120 is a viral envelop glycoprotein and its interaction with chemokine receptors (CXCR4, CCR5) and potentially with PrP^C-NOX pathway, was described to stimulate cofilin-actin rod formation in neurons (Figure 10) (Smith et al., 2021). Genetic ablation of PrP^C or NOX, as well as administration of CXCR4/CCR5 antagonists (AMD3100/maraviroc, respectively), was sufficient to inhibit cofilin-actin rod formation induced by gp120 (Smith et al., 2021). Interestingly, the same report found that cofilin-actin rods induced by Aβ dimers/trimers ($Aβ_{d/t}$) also occurred through a CXCR4/CCR5 pathway since the use of the chemokine receptors inhibitors (AMD3100/maraviroc) rescued $Aβ_{d/t}$-induced rod formation. In support of these findings, an increased number of reports have been implicating CCR5 in AD pathogenesis (Li and Zhu, 2019). Regarding CXCR4, the administration of AMD3100 (CXCR4 antagonist) to a transgenic mouse model of AD improved neuroinflammation, cognition and pathophysiology markers (Gavriel et al., 2020). This suggests that in addition to the described PrP^C-NOX pathway, chemokine receptors might also be implicated in cofilin dysregulation in AD. Together, these findings support that rod formation might occur through similar pathways in different diseases, including HAND and AD.

To summarize, there are several signaling pathways that appear to be involved in rod formation and the crosstalk between these pathways should be carefully interpreted to properly elicit the molecular mechanisms underlying rod formation.

2.1.1.2 Cofilin pathology in neurodegenerative diseases

Cofilin-actin rods have been thoroughly studied in AD, where they were identified in neurons treated with Aβ oligomers, in AD mouse models and in *postmortem* brains from AD patients (Minamide et al., 2000; Maloney et al., 2005; Rahman et al., 2014). In fact, in AD brains, the majority of dense core amyloid plaques were found associated with rod-like aggregates (Minamide et al., 2000). Interestingly, in cultured neurons, rods are formed in response to Aβ oligomers or $Aβ_{d/t}$, being the last one forms much more potent as rod inducers (Minamide et al., 2000; Cleary et al., 2005; Maloney et al., 2005; Davis et al., 2009; Mc Donald et al., 2010; Davis et al., 2011). Currently, it is known that Aβ overproduction and oxidative stress induce cofilin-actin rods formation in AD (Bamburg et al., 2010). In turn, the formation of cofilin-actin rods triggers the blockage of the cellular transport of several

organelles and vesicles including the Amyloid precursor protein (APP)-containing vesicles, providing a local production of Aβ (Maloney et al., 2005). Therefore, the increased levels of Aβ lead to cofilin-actin rods formation representing a feed-forward mechanism (Maloney et al., 2005). In AD brains, cofilin-actin rods have been mainly found in frontal cortex and hippocampal regions corroborating the impact of rod formation in synaptic dysfunction and cognitive impairment in AD (Minamide et al., 2000).

Although rods, which require cofilin dephosphorylation and activation, were highly reported in AD, some studies showed that cofilin phosphorylation (inactivation) might also contribute to AD pathogenesis. For instance, in the postsynaptic density (PSD) fraction from cortical samples of an AD mouse model and AD human brains, phosphorylated cofilin was increased (Rush et al., 2018). Moreover, short incubation of primary cortical neurons with Aβ oligomers promoted cofilin phosphorylation and F-actin stabilization in dendritic spines affecting synaptic function (Rush et al., 2018). Interestingly, the use of the ROCK inhibitor fasudil, prevented F-actin stabilization and synaptic dysfunction induced by Aβ oligomers. Specifically, ROCK inhibition was suggested to mediate LIMK inactivation and consequent inhibition of cofilin phosphorylation (Rush et al., 2018).

Collectively, the current literature suggests that the signaling pathways that regulate cofilin activity and consequent actin dynamics are greatly involved in the synaptic dysfunction and cognitive deficits observed in AD patients. This hypothesis is strongly supported by a report showing that the genetic reduction of *cofilin* is sufficient to rescue APP/Aβ-induced synaptic protein loss and deficits in LTP and contextual memory in an AD mouse model (Woo et al., 2015b).

Regarding HD, the formation of cofilin-actin rods was also reported, although with a nuclear localization (Munsie and Truant, 2012). Cofilin has been described as required to the nuclear translocation of actin (Dopie et al., 2012). Under stress conditions, the large influx of cofilin and actin into the nucleus might lead to cofilin-actin rods formation. How this reflects in the development of HD is still unexplored, however, it was reported that wild type huntingtin directly stimulated cofilin-actin rods formation during heat shock stress response, supposedly to preserve ATP levels necessary for other cell functions (Munsie et al., 2011).

Cofilin-actin rods were also associated with ischemic stroke, where acute cerebral blood flow reduction results in neuronal cell death in the infarct area and neuronal damage of the surviving neurons in the peri-infarct area. In a rat model of ischemic stroke, cofilin-actin rod formation was observed in the peri-infarct area leading to mitochondrial transport impairment and synaptic dysfunction (Won et al., 2018; Shu et al., 2019). Corroborating a key role of cofilin in the pathology, the overexpression of LIMK1 and consequent inactivation of cofilin was sufficient to rescue ischemia-induced cofilin-actin rod formation and to protect synaptic function in both cultured neurons and in the ischemic rat model (Shu et al., 2019;

Chen et al., 2020).

Concerning HAND, cofilin-actin rods were observed in primary cultures of hippocampal neurons treated with the viral envelop glycoprotein gp120 (Smith et al., 2021). Although the relevance of rods for the disease progression needs further investigation *in vivo*, it was suggested a possible involvement of cofilin dysregulation in synaptic degeneration and cognitive decline in the disease (Smith et al., 2021).

In respect to PD, the dysregulation of cofilin was also observed. It was shown that parkin, a protein which mutations are associated with inherited cases of PD, was able to interact with LIMK inhibiting its ability to phosphorylate and inactivate cofilin (Lim et al., 2007). Additionally, αSyn was shown to induce cofilin phosphorylation (inactivation) and actin stabilization in primary neurons and in fibroblasts-derived neurons from PD patients (Bellani et al., 2014). A more recent report using a *Drosophila* model with αSyn overexpression, showed that αSyn interacts with the ABP spectrin, thereby altering actin dynamics (Ordonez et al., 2018). This culminated in the mislocalization of the mitochondrial fission protein Drp1 leading to mitochondrial dysfunction and neuronal death (Ordonez et al., 2018). In this study, one of the reported actin dysfunctions was the formation of cofilin-actin rods. Nevertheless, the potential involvement of cofilin-actin rods in PD is still unexplored.

3 Synucleinopathies

Synucleinopathies comprise a group of diseases which arise from the intracellular accumulation of abnormal filamentous material majorly composed of misfolded αSyn (Polymeropoulos et al., 1997; Spillantini et al., 1997; Spillantini et al., 1998b). These diseases include PD, Dementia with Lewy Bodies (DLB), Multiple System Atrophy (MSA) and Pure Autonomic Failure (PAF) (Papp et al., 1989; Kaufmann, 1996; Spillantini et al., 1998a; Spillantini and Goedert, 2000). αSyn-containing inclusions are the neuropathological hallmark of these diseases and these inclusions comprise Lewy Bodies (LBs), present in the cell bodies of affected neurons, Lewy Neurites (LNs), formed in the neurites of diseased neurons and Glial Cytoplasmic Inclusions (GCIs), developed in the cell bodies of oligodendrocytes in MSA. The distinct strains of αSyn inclusions and the cellular population affected in each disease contributes to the differences observed in the clinical diagnosis. However, synucleinopathies also share some symptoms such as decline in motor function, cognitive impairment, and behavioral and autonomic dysfunctions, which can vary in penetrance according to each disease. For instance, PAF is characterized by an extensive failure of the autonomic system which is accompanied by LBs and LNs in the peripheral nerves (Kaufmann, 1996). Nevertheless, in PAF, αSyn inclusions can also be detected in the central nervous system (CNS) and the disease can progress to PD, DLB or MSA (Arai et al., 2000).

Considering the unknown etiology of sporadic synucleinopathies, the susceptibility to develop disease seems to arise from a combination of factors including genetic predisposition, lifestyle, environmental exposure and age, with αSyn misfolding and inclusions playing a central role in the progression of these diseases.

3.1 αSynuclein

The synuclein family includes three synuclein proteins (α-, β-, and γ-) and they were first identified through several independent lines of research, namely in *Torpedo californica* electric organ, associated with synaptic vesicles (Maroteaux et al., 1988; Maroteaux and Scheller, 1991), in bovine and songbirds, as a brain specific protein (Nakajo et al., 1990; George et al., 1995), and in amyloid plaques, as a non-amyloid component (Ueda et al., 1993). Subsequently, there was the identification of the human homologues α- and β-synucleins (Jakes et al., 1994). The name “synuclein” was based on the initial findings of its synaptic and nuclear localization. Although synucleins are ubiquitously expressed and can be detected in several tissues they are highly enriched in the nervous system being α-

and β-synuclein predominant in CNS and γ-synuclein prevalent in peripheral nervous system (Jakes et al., 1994; Lavedan et al., 1998). As the focus of the current work is α-Synuclein (αSyn), it will be referred hereafter, and the other family members will be mentioned only when justified.

Gene, protein and structure

The human αSyn encoding gene (*SNCA*) contains 114 kb and is located at chromosome 4q21 (Chen et al., 1995; Spillantini et al., 1995). *SNCA* is composed of 6 exons with the last 5 exons constituting the coding region, as the initiation codon (ATG) is located in exon 2, and the termination codon (TAA) is located in exon 6 (Spillantini et al., 1995; Xu et al., 2015). The main transcript originates αSyn which is a small protein composed of 140 amino acids comprising three different structural motifs (Figure 11) (Jakes et al., 1994). The N-terminus is composed by seven repeats of the consensus sequence KTKEGV, which comprises the lipid-binding domain of the protein. Membrane binding increases the stabilization of the protein structure due to the increased α-helical conformation. Although αSyn associates with membranes, its sequence lacks a lipid anchor or transmembrane domain. This is also the region where mutations liked to familial forms of synucleinopathies have been described (Figure 11), and it was suggested that these mutations contribute to the mislocalization of αSyn (Jensen et al., 1998; Bussell and Eliezer, 2004). The central region is composed by an hydrophobic non-amyloid β component (NAC) domain, which contributes to the conformational change of αSyn towards a β-sheet enriched structure, promoting αSyn aggregation and toxicity (Ueda et al., 1993). The negatively charged carboxy-terminal region is enriched in acidic and proline residues and interacts with several proteins and partners including Ca^{2+} (Alderson and Markley, 2013). This region seems to be important to decrease protein aggregation as truncated forms of αSyn lacking the C-terminal region are more prone to aggregate (Murray et al., 2003).

Structurally, αSyn is monomeric and natively unfolded in solution adopting an α-helical conformation when bound to high curvature membranes and/or acidic phospholipid headgroups-enriched membranes (Eliezer et al., 2001; Jao et al., 2004). Additional studies have reported an organized folded tetramer of αSyn, which was considered to be resistant to oligomerization (Bartels et al., 2011; Wang et al., 2011).

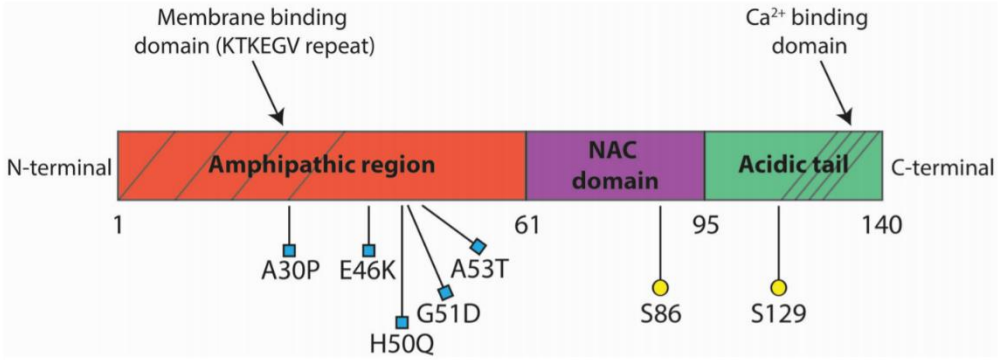

Figure 11 – Schematic representation of αSyn protein regions. Red: N-terminal domain composed by the KTKEGV repeats which consists of the membrane binding domain of αSyn and is the region where human missense mutations associated to familial PD have been found. Purple: central hydrophobic region named NAC domain which contributes to the aggregation of the protein. Green: C-terminal domain composed of acidic residues; constitutes the binding site for several proteins and partners. Taken from (Emanuele and Chieregatti, 2015).

Function

αSyn is highly expressed in the nervous system and under physiological conditions is concentrated in nerve terminals and found in close proximity with synaptic vesicles (Maroteaux and Scheller, 1991; Jakes et al., 1994). During neuronal maturation αSyn is one of the latest proteins to be targeted to the synapse, suggesting a function in later stages of synapse formation or in synapse maintenance (Withers et al., 1997). Once in the pre-synaptic terminals, αSyn was shown to associate with synaptic vesicles, interact with pre-synaptic proteins and serve as a SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) chaperone essential for vesicle fusion (Burre et al., 2010; Chen et al., 2013; Zaltieri et al., 2015). Considering this, even though the precise function of αSyn is still a matter of debate, one of the most relevant physiologic functions appears to be in regulating synaptic homeostasis and neurotransmitter release. In accordance with this, studies with αSyn knockout (KO) mice showed normal basal hippocampal synaptic function, but under challenge conditions where docked and reserve pool vesicles were exhausted, significant synaptic impairment was evident (Cabin et al., 2002). Additionally, αSyn KO mice presented slower refilling of vesicles from the reserve pool to the docked pool, suggesting that αSyn contributes to synaptic vesicles trafficking and maintenance (Abeliovich et al., 2000; Cabin et al., 2002). Aiming at clarifying if the other synuclein proteins have a compensatory role in the αSyn KO, a triple KO mouse lacking the three synucleins was developed. The loss of the three synucleins did not result in decreased number of midbrain dopamine neurons although contradictory findings were reported since the single KO (αSyn KO) mice showed reduced number of dopaminergic neurons (Anwar et al., 2011; Garcia-

Reitboeck et al., 2013). Triple KO mice showed hyperactivity but not parkinsonism and with age these mice died prematurely, developed visual impairments and showed alterations in presynaptic morphology; however, no changes in synapse or neuronal number were reported (Greten-Harrison et al., 2010; Anwar et al., 2011). Although loss of all synucleins had little effect on excitatory neurotransmission, triple KO mice showed decreased SNARE-complex assembly and altered synaptic structure and size which were accompanied by memory deficits and learning impairment (Burre et al., 2010; Greten-Harrison et al., 2010). The role of αSyn at the synapse was reinforced by a report where αSyn overexpression recovered the SNARE-complex assembly defects, lethality and neurodegeneration caused by the deletion of cysteine-string protein-α (CSPα) in mice (Chandra et al., 2005). Altogether, the current knowledge suggests that αSyn may not be vital for the basic machinery of synaptic transmission but certainly contributes to the long-term maintenance of synaptic functions.

In addition to the synapse, several studies have reported other localizations for αSyn such as to the mitochondria, nucleus, endoplasmic reticulum (ER), Golgi and cytoskeleton (Burre et al., 2018). Most of these observations were based on αSyn overexpression studies questioning the physiologic significance of the findings. Nonetheless, these interactions may be relevant in αSyn pathophysiology.

3.2 αSynuclein pathogenesis

αSyn dysregulation is a major contributor to neurodegeneration since missense mutations and multiplications of the *SNCA* gene are linked to familial cases of disease, causing severe forms of synucleinopathies (Polymeropoulos et al., 1997; Kruger et al., 1998; Singleton et al., 2003). Although familial cases represent a small percentage of patients, it is undoubtable the involvement of αSyn in disease progression, either in familial or sporadic cases, since αSyn inclusions (LBs) are the defining neuropathological hallmark of *postmortem* diagnosis of synucleinopathies.

Aggregation

αSyn is mainly found in fibrillar aggregates in LBs, the neurodegeneration hallmark; however, several evidences indicated that oligomeric and small fibrillar αSyn species are responsible for causing neuronal degeneration in synucleinopathies and largely contributing for the progression of pathology (Lee et al., 2005; Winner et al., 2011). αSyn oligomerization is a nucleation dependent process which starts with changes in protein conformation from unfolded to partially folded oligomers. This conformational change allows the exposure of

the NAC region which promotes aggregation through hydrophobic interactions. Upon nuclei formation αSyn monomers are rapidly added leading to the generation of large oligomers, protofibrils and fibrillar aggregates (Figure 12) (Li et al., 2001). αSyn oligomeric species are one of the most toxic species causing neuronal degeneration, however, these are intermediate transient species thought to precede the fibrillary aggregates, making it challenging to assess its pathological impact (Winner et al., 2011; Helwig et al., 2016). The mechanisms involved in αSyn oligomerization/aggregation are influenced by several factors including αSyn mutations, expression levels, clearance efficiency, PTMs, metals concentration, and environmental agents (e.g. pesticides), among others (Li et al., 2001; Lee et al., 2005; Bras et al., 2020). As mentioned above, αSyn appears to localize to several organelles although without a clear physiologic role; however, αSyn aggregation disturbs the homeostasis of those organelles leading to the impairment of several physiological processes which include: synaptic vesicle trafficking, mitochondrial activity, ER-Golgi function, plasma membrane integrity, protein clearance system function, and inflammatory responses (Alam et al., 2019; Bras et al., 2020). In this respect, it is still a matter of debate whether αSyn pathology arises from a loss of physiologic function or a gain of toxic function, most likely, it is a consequence of both.

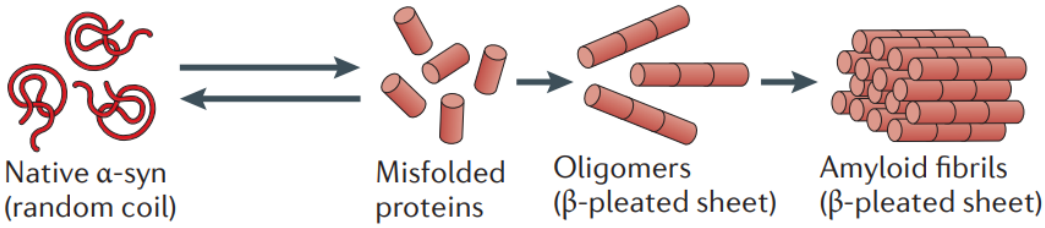

Figure 12 – Depiction of the model of αSyn aggregation. αSyn aggregation cascade under pathological conditions. Adapted from (Irwin et al., 2013).

αSyn post-translation modifications

αSyn can undergo several PTMs which also play a role in pathogenesis. The PTMs mostly implicated in pathology include phosphorylation, nitration and oxidation. Phosphorylation is one of the most relevant modifications since the phosphorylation of the residue S129 (αSyn pS129) is found in more than 90% of the αSyn present in LBs, and only 4% present in soluble αSyn, being an hallmark of synucleinopathies (Fujiwara et al., 2002; Anderson et al., 2006). Although this modification was reported to increase the rate of αSyn fibrillization *in vitro*, the specific function of this modification remains unclear. Many studies have focusing on αSyn S129 phosphorylation and *in vitro* and cell-based studies have led to the identification of several kinases promoting this phosphorylation, namely the

casein kinase I and II, G-protein-coupled receptors (GPCRs), leucine-rich repeat kinase 2 (LRRK2) and polo-like kinases (Plk) (Okochi et al., 2000; Pronin et al., 2000; Inglis et al., 2009; Qing et al., 2009). αSyn can be also phosphorylated on residues S87, Y125, Y133 and Y136; although these modifications are thought to suppress αSyn aggregation, their role is far less explored and only a function for S87 phosphorylation has started to be unraveled. It has been suggested that αSyn phosphorylation at S87 maintains αSyn in an unfolded state avoiding its fibrillization and reducing its membrane binding (Paleologou et al., 2010).

Oxidative stress plays an important role in αSyn pathology as it can promote αSyn nitration or oxidation potentiating aggregation (Souza et al., 2000; Takahashi et al., 2002). Although αSyn nitration and oxidation have been implicated in synucleinopathies, it is unclear whether they represent a primary event resulting in αSyn aggregation or whether they are a product of the reaction of the reactive species with the small aggregates, propagating αSyn pathology and contributing to disease progression. A number of other αSyn PTMs have been described including SUMOylation, acetylation, ubiquitination, glycation, glycosylation and proteolysis; however, their functional significance needs further analysis as in some cases controversial results have been reported, and it is important to clarify which PTMs have a physiologic and/or a pathologic relevance (Burre et al., 2018).

SNCA mutations and multiplications

SNCA mutations were the first genetic evidence of a link between αSyn and pathology. Until now there are six mutations described to cause familial forms of synucleinopathy with high penetrance, namely A30P, E46K, A53T/E, H50Q and G51D (Polymeropoulos et al., 1997; Kruger et al., 1998; Capani et al., 2001; Zarranz et al., 2004; Appel-Cresswell et al., 2013; Kiely et al., 2013; Ghosh et al., 2014). All these mutations are single amino acid changes occurring in the same protein region and leading to early onset synucleinopathies. However, each mutation exerts different outcomes regarding αSyn aggregation and rate of disease progression. For instance, the most potent mutation known so far is G51D which was described to lead to the earliest disease onset (Rutherford et al., 2014). Patients carrying A30P usually have a late onset and mild form of dementia whereas patients harboring the A53T mutation are affected by a severe form of parkinsonism often associated with dementia (Kruger et al., 1998; Spira et al., 2001). Regarding the effect of the mutations in αSyn aggregation, while G51D, A30P and A53E appeared to slow down the rate of fibril formation, E46K, H50Q and A53T mutations led to an increased rate of fibril formation (Polymeropoulos et al., 1997; Kruger et al., 1998; Greenbaum et al., 2005; Ghosh et al., 2013; Ghosh et al., 2014). As already mentioned, in general these mutations result in

reduced phospholipid binding indicating that αSyn physiologic function can become compromised in the early onset mutants.

Besides *SNCA* mutations, several studies have showed that increased levels of αSyn expression, driven by *SNCA* duplications and triplications, are sufficient to cause synucleinopathies (Singleton et al., 2003; Chartier-Harlin et al., 2004). Interestingly, while *SNCA* duplications lead to a mild phenotype, gene triplications exhibit a more severe phenotype, implying a direct correlation between gene dosage and pathology (Muenter et al., 1998; Farrer et al., 2004; Obi et al., 2008). Importantly, multiplications of the *SNCA* gene have also been associated with increased penetrance of cognitive impairment and dementia in synucleinopathy patients. Given the importance of the increased intracellular concentrations of αSyn in promoting pathology, it is conceivable that αSyn may also play a role in sporadic synucleinopathies. In accordance with this, polymorphisms in noncoding regions of the *SNCA* gene were described as major risk factors for sporadic disease (Simon-Sanchez et al., 2009).

Prion-like spreading of αSyn pathology

αSyn was considered an intracellular protein for a long time, but this idea was challenged after the detection of αSyn in extracellular fluids such as blood plasma and cerebrospinal fluid (CSF) in both healthy subjects and PD patients (Borghi et al., 2000; El-Agnaf et al., 2003). Nevertheless, the amount of αSyn secretion was not clearly correlated with pathogenesis as both elevation and reduction of exogenous αSyn levels were reported in PD patients (Anderson et al., 2006; Tokuda et al., 2010). Considering this, it is still unclear whether αSyn levels in biological fluids can be used as a biomarker of disease. Knowing that αSyn is present exogenously, increasing evidence support the idea that αSyn might act as a pathogen responsible for the spreading of pathology (Figure 13). In line with this, αSyn pathology can develop by a pattern of LB pathology transmitted between neurons, starting in enteric nervous system, spreading to the brainstem nuclei, then to the midbrain, and ultimately to cortical areas (Braak et al., 2004). This mechanism may explain the progression of PD beyond the most obvious motor symptoms, including depression, dementia, and autonomic and sensory dysfunction, as will be further discussed. This pattern of self-propagation of diseased-related proteins resembles the one of prion diseases. In fact, the prion-like propagation hypothesis was raised by findings with *postmortem* studies of PD patients, who died 11-16 years after received a fetal brain tissue transplantation and presented αSyn-positive LB pathology in the grafted neurons (Kordower et al., 2008; Li et al., 2008). This hypothesis opened a new field of research aiming at investigating the spreading of αSyn pathology. To address this, several experimental setups were designed,

and the initial studies demonstrated that in a PD mouse model, which was engrafted with neuronal precursor cells, αSyn pathology was transmitted from the affected neurons to the engrafted cells, corroborating the cell-to-cell transmission of αSyn pathology (Desplats et al., 2009). Additional *in vitro* studies confirmed this hypothesis, which showed that exogenous recombinant αSyn fibrils induced intracellular αSyn pathology by promoting aggregation of the endogenous protein (Luk et al., 2009). Further corroborating these observations, brain homogenates from old TgM83 transgenic mice (expressing human A53T αSyn) injected in young TgM83 transgenic animals resulted in accelerated αSyn pathology and disease progression (Mougenot et al., 2012). In order to disclose that disease progression in the described *in vivo* experiments arises from αSyn pathology and not from unknown host factors, in the last decade an increased number of reports have been using recombinant αSyn pre-formed fibrils (PFFs), which result from the sonication of αSyn fibrils (Chung et al., 2020). The inoculation with αSyn PFFs, *in vitro* or *in vivo*, resulted in protein aggregation, neuroinflammation, neuronal loss and behavioral deficits, recapitulating several core features of synucleinopathies (Luk et al., 2012a; Luk et al., 2012b; Sacino et al., 2013; Osterberg et al., 2015; Kim et al., 2019; Chung et al., 2020). Remarkably, these short fibril fragments showed a strong pathologic effect that was attributed to their seeding and spreading capacities resulting in widespread αSyn pathology resembling human PD (Alam et al., 2019; Froula et al., 2019). In addition to the inoculation with *in vitro* generated αSyn PFFs, the extraction and injection of αSyn derived from brains of PD, DLB or MSA patients, in several animal models, including mice and monkeys, induced protein aggregation and pathology, further establishing the prion hypothesis for αSyn (Masuda-Suzukake et al., 2013; Recasens et al., 2014; Pieri et al., 2016).

Overall, these studies support the conceptual breakthrough that misfolded αSyn behaves as the spreading agent leading to the prion-like propagation of the disease in a cell non-autonomous manner.

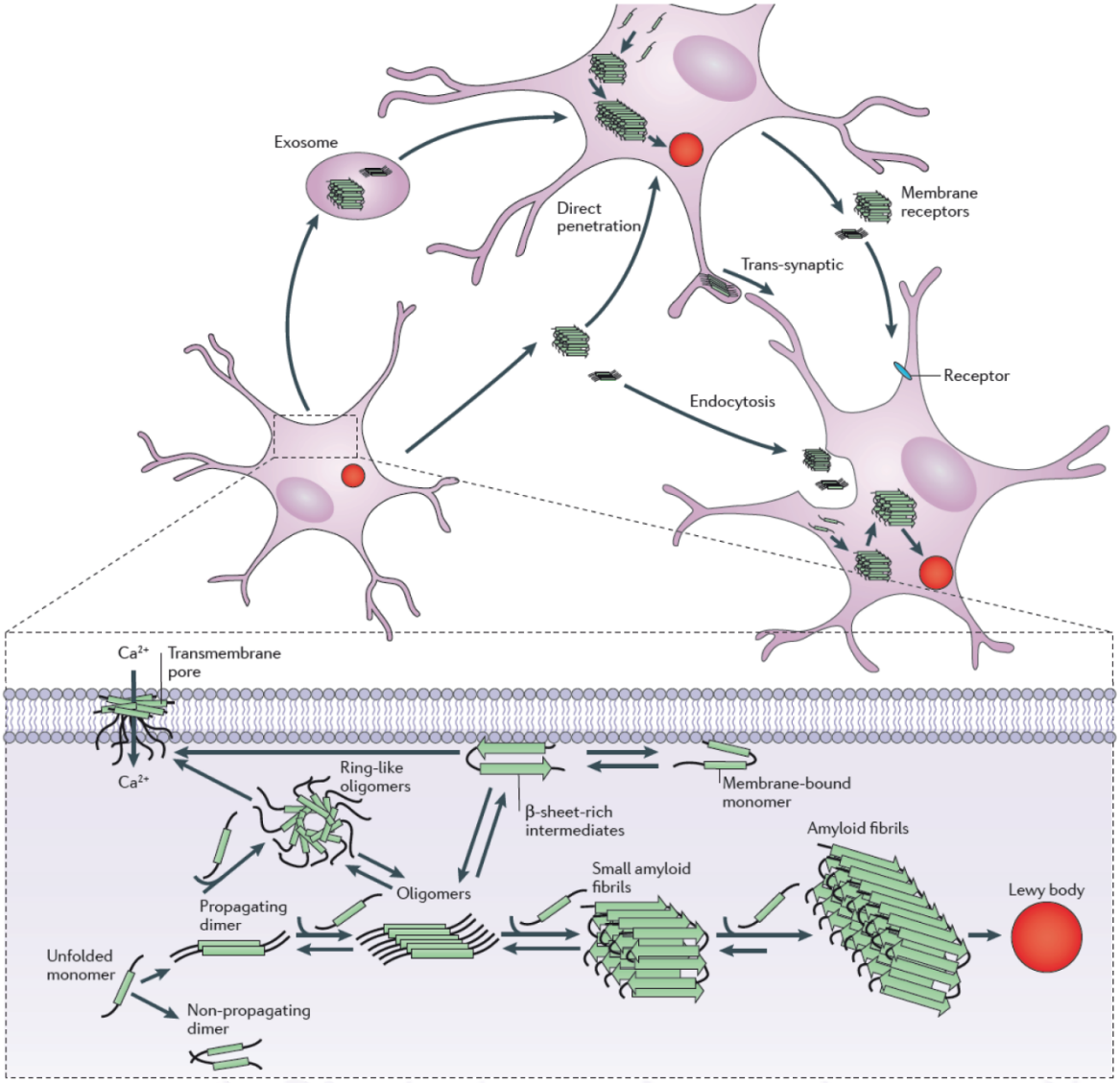

Figure 13 – Schematic representation of the mechanisms of αSyn aggregation and spreading. αSyn aggregated species, comprising oligomers and fibrils, can be transmitted between neurons and induce the aggregation (seeding) of the endogenous αSyn in the recipient cells. The mechanisms through which αSyn is transferred between neurons can be multiple including endocytosis, disruption of the cell membrane and direct penetration, membrane receptors or trans-synaptic transmission. Taken from (Lashuel et al., 2013).

3.3 Parkinson's Disease

PD was first described by James Parkinson more than 200 years ago, and nowadays accounts for the second most common neurodegenerative disease in the world, after AD (Parkinson, 2002; Pringsheim et al., 2014). Many advances have been made towards the understanding of the disease, yet many of James Parkinson's observations remain cardinal features of PD. PD is typically characterized by motor symptoms which range from resting tremors, rigidity, slowness of movement to postural instability. Pathologically, PD develops as a result of intracellular LBs accumulation and consequent loss of dopaminergic neurons

in the substantia nigra pars compacta (SNpc), leading to dysregulation of basal ganglia activity (Dauer and Przedborski, 2003). The loss of dopaminergic neurons results in striatal dopamine deprivation originating most of the motor symptoms (Dauer and Przedborski, 2003). Nevertheless, neuronal loss was also reported in other brain regions in PD such as locus coeruleus, raphe nucleus, amygdala, hypothalamus, among others (Braak et al., 2003b; Halliday et al., 2011). In fact, the progression of LB pathology in PD was investigated by Braak and his colleagues, who proposed a staging system in which αSyn pathology starts in the olfactory mucosa and/or enteric nervous system, progressing to the brain through vagal and olfactory nerves, and gradually reaching several regions of the brain namely the pontine tegmentum, midbrain, mesocortex and allocortex and lastly neocortical regions (Braak et al., 2003a; Braak et al., 2003b; Braak et al., 2004).

Genetic risk factors

PD is a progressive neurodegenerative disorder and about 90% of the cases are sporadic. In the remaining cases the disease is inherited indicating that it has a genetic cause which arises from mutations or variations in several genes including, but not limited to, *SNCA* (αSyn), *LRRK2*, *GBA* (glucocerebrosidase), *MAPT* (microtubule-associated protein tau), *PINK1* (PTEN-induced kinase 1)*, DJ-1* (protein deglycase, also known as PARK7), and *PRKN* (parkin), among others, or from *SNCA* duplications or triplications (Neudorfer et al., 1996; Polymeropoulos et al., 1997; Hutton et al., 1998; Kitada et al., 1998; Kruger et al., 1998; Bonifati et al., 2003; Singleton et al., 2003; Zimprich et al., 2004; Halperin et al., 2006).

Non-motor symptoms

Besides motor symptoms, PD patients also develop a variety of non-motor symptoms which include rapid eye movement (REM) sleep disorder, olfactory dysfunction, constipation, hyposmia, apathy, depression, anxiety and cognitive impairment, which may precede motor symptoms (Jellinger, 2015; Titova et al., 2017). In some patients, cognitive impairment evolves to dementia in later stages of the disease progression leading to Parkinson's disease dementia (PDD).

3.4 Lewy Body dementias

Cognitive impairment and dementia are established features of Lewy Body dementias (LB dementias), which include Parkinson's disease dementia (PDD) and Dementia with Lewy Bodies (DLB). As these diseases present very similar clinical and pathological features, some debate has been raised on whether these diseases should be considered

only as one (Friedman, 2018). Actually, when parkinsonism is present and the clinical picture is fully developed both diseases are indistinguishable (Friedman, 2018). However, PDD and DLB are clinically considered two diseases and they are usually distinguished by the time at which dementia is identified (Irwin et al., 2013). International consensus has defined that DLB is diagnosed when cognitive impairment precedes parkinsonism within 1 year, whereas PDD is diagnosed when cognitive impairment develops after 1 year of parkinsonism diagnosis (McKeith et al., 2005; Emre et al., 2007). At the disease diagnosis it is estimated that around 30% of PD patients already present some degree of cognitive impairment and during disease progression is predicted that around 80% of PD patients develop dementia (Aarsland et al., 2003; Halliday et al., 2008; Kempster et al., 2010). DLB accounts for the second most common dementia after AD, and it is likely that the prevalence of DLB is underestimated due to misdiagnosis (Burn et al., 2003). As LBs accumulation and cognitive decline and dementia are hallmarks of PDD and DLB, these diseases can be grouped in Lewy body dementias (LB dementias).

Due the similarities between PDD and DLB, the molecular mechanisms underlying these diseases are most likely overlapping, thus the two diseases will not be distinguished henceforth. LB dementias are very debilitating diseases and the mortality associated is three times higher than normal population (Larsson et al., 2018). The core features of LB dementias are cognitive decline, parkinsonism (does not always occur in DLB), fluctuating levels of alertness, visual hallucinations and REM sleep disorder (Jellinger, 2015). Dementia is usually preceded by cognitive impairment and the clinical features differ somehow from those present in AD. The cognitive profile in LB dementias is characterized by impairment of executive functions comprising planning, complex thinking and mental flexibility (Petrova et al., 2012). These patients exhibit early and pronounced impaired attention and visuo-spatial functions and common behavioral symptoms (Petrova et al., 2012). Regarding behavioral symptoms, it is described that at least one neuropsychiatrist symptom is present in more than 90% of patients suffering with synucleinopathies which can include apathy, depression, hallucinations, delusions, anxiety and insomnia (Aarsland et al., 2007). Although memory is not impaired to the extent observed in AD patients, LB dementia patients also present deficits in working memory, visual and verbal memory as well as in learning (Weintraub et al., 2004; Hanagasi et al., 2017). Additionally, autonomic dysfunctions are also frequent in these patients (Mendoza-Velasquez et al., 2019). The plethora of non-motor symptoms that patients may develop are very debilitating contributing to a considerable detriment of the quality of life of the patients and to the increase of the care given burden.

Hippocampal and cortical pathology

PD patients have LBs restricted to the brainstem region and the caudal-rostral progression of these lesions to the limbic and neocortical areas correlates with the clinical dementia developed in patients with LB dementias (Braak et al., 2003a). Cognitive impairment in synucleinopathies can be related with dysfunction of the brain regions responsible for cognition namely the cortex and the hippocampus which, actually, are major sites of αSyn expression (Figure 14) (Maroteaux and Scheller, 1991). The hippocampus belongs to the limbic system and is one of the main regions involved in the consolidation of new memories, spatial orientation, navigation and emotional responses (Lavenex et al., 2007). The entorhinal cortex is considered to belong to the hippocampal region as it connects the hippocampus with the other brain regions. The hippocampus appears to be one of the most vulnerable regions in synucleinopathies, and hippocampal dysfunction is likely to provoke the several cognitive and behavioral symptoms described above for LB dementias (Lauterbach, 2004; Adhikari et al., 2010; 2011). In this respect, hippocampal volume loss was observed in patients with LB dementias, which correlated with decreased cognitive impairment, and was also associated with high depression levels (Camicioli et al., 2003; Tam et al., 2005; Bouchard et al., 2008; Chen et al., 2015a). Neuroimaging evidences showed that hippocampal atrophy was usually present in LB dementias patients with shorter survival (Seibert et al., 2012; Graff-Radford et al., 2016).

Considering αSyn accumulation in LBs as a hallmark of disease, it is expected that these inclusions are present and contribute to hippocampal/cortical pathology promoting the observed clinical symptoms. In light of this, LB dementia patients showed increased density of αSyn inclusions in several regions including the amygdala, hippocampus and entorhinal cortex (Armstrong et al., 2014; Adamowicz et al., 2017). Furthermore, the longer the disease the more pronounced was the accumulation in the limbic and cortical regions culminating in severer cognitive impairment (Apaydin et al., 2002). Additional reports found that high LBs density in limbic regions could be sufficient to distinguish between cognitively impaired and non-cognitively impaired patients with accuracy (Harding and Halliday, 2001). Corroborating this, other studies suggested that LBs accumulation in limbic areas could be fundamental for the development of dementia in LB dementias (Kovari et al., 2003).

Together, the current knowledge points to a major involvement of the hippocampal and cortical αSyn pathology for the development of cognitive impairment and dementia in synucleinopathies.

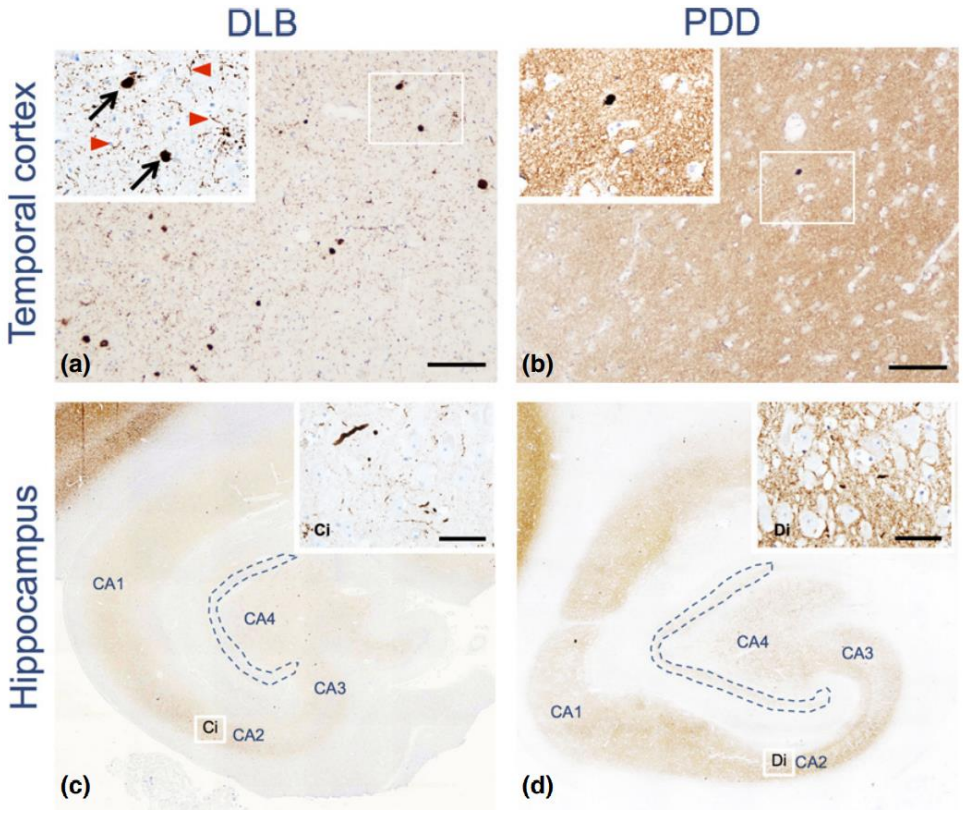

Figure 14 - Histological analyses of the temporal cortex and hippocampus of LB dementia cases. Lewy body pathology, namely Lewy Bodies (black arrows) and Lewy Neurites (red arrowheads), is present in DLB, (Dementia with Lewy Bodies) and PDD (Parkinson's Disease Dementia). Taken from (Walker et al., 2019).

αSyn-induced synaptic dysfunction and dementia

It is unquestionable that LB pathology in cortical/hippocampal regions is directly correlated with cognitive decline, however, several reports have stated that LBs might be formed as a protective cellular mechanism to confine the pathogenic forms inhibiting their circulation (Karpinar et al., 2009; Chen et al., 2015b). LBs are considered to constitute the final stage of a pathological cascade in which intermediate oligomers and small aggregates represent the most toxic species, as aforementioned. In accordance with this, several reports, with DLB patients, described an initial aggregation and accumulation of αSyn at the pre-synapse resulting in synaptic dysfunction and triggering the neurodegeneration process (Kramer and Schulz-Schaeffer, 2007; Schulz-Schaeffer, 2010; Adamowicz et al., 2017). Moreover, αSyn-induced synapse loss was associated with dementia severity in later stages of disease progression (Ubhi et al., 2010). Interestingly, this suggests that pathology may start at the synapse and progress to the cell body, contributing to the observed neuronal dysfunction and preceding neuronal death. In this respect, a significant amount of αSyn aggregates was observed throughout the cortex of LB dementia patients, localized at the synapses, and the amount of these aggregates exceeded the one of mature LBs or LNs (Kramer and Schulz-Schaeffer, 2007; Schulz-Schaeffer, 2010). Additional analysis of synaptic markers in LB dementia cases showed a considerable reduction of presynaptic

markers and structures (Kramer and Schulz-Schaeffer, 2007; Revuelta et al., 2008; Nikolaus et al., 2009). As dendritic spines are closely related with synaptic activity, when looking at postsynaptic markers, LB dementia cases presented a reduction of the postsynaptic density protein 95 (PSD-95) and the ABP drebrin, accompanied by a dramatic reduction of dendritic spines (Zaja-Milatovic et al., 2006; Kramer and Schulz-Schaeffer, 2007; Schulz-Schaeffer, 2010). Similar findings were reported for striatal regions and substantia nigra in PD patients, suggesting a common pathophysiological mechanism at the synapses in which αSyn aggregation may lead to the clinical symptoms observed in synucleinopathies (Zaja-Milatovic et al., 2005; Deutch, 2006).

Complementary risk factors for dementia

An important contributor to cognitive decline in LB dementias is the AD-related pathology since up to 30% of the cases were shown to present hyperphosphorylated tau and Aβ accumulations, which usually appear at later stages of disease progression (Hepp et al., 2016). Thus, LB dementias are heterogeneous diseases and although the relative contribution of each pathology is still unclear, the concomitant pathologies have been associated with accelerated decline in cognition and shorter survival time (Ruffmann et al., 2016).

Although LB dementias are primarily sporadic diseases, several studies have focused in identifying genetic risk factors for these diseases. Apolipoprotein epsilon 4 (ApoE ε4) was identified as a risk factor for LB dementias even in the absence of clear AD pathology (Tsuang et al., 2013; Bras et al., 2014; Guerreiro et al., 2016; Guerreiro et al., 2018; Sun et al., 2019). Mutations in GBA encoding gene have also been described to account for an increased risk for developing not only PD but also early-onset dementia (Nalls et al., 2013; Zokaei et al., 2014; Brockmann et al., 2015). As expected, the *SNCA* locus is a main hit in PD, and *SNCA* mutations have been linked with cognitive decline and consequent LB dementia development (Morfis and Cordato, 2006; Papadimitriou et al., 2016). Besides mutations, duplications and triplications of the *SCNA* gene have a robust association with dementia although with phenotypical variations and clinical heterogeneity (Fuchs et al., 2007; Mata et al., 2014).

Therapeutic approaches

The treatment of motor symptoms in PD is typically achieved with the use of dopamine precursors, namely levodopa, however, there are potential side effects including neuropsychiatric symptoms and cognitive decline (Beaulieu-Boire and Lang, 2015). Currently, there are no disease-modifying therapies for LB dementias; however, due to the reduction in cholinergic markers in these diseases, the use of cholinesterase inhibitors

(ChEIs) is the only treatment available which demonstrated a modest efficacy in hampering the cognitive symptoms (Chow, 2005; Meng et al., 2019). Although hopeful results have been described, these strategies are symptomatic therapies without evidencing a protective effect on neurodegeneration. Thus, new therapeutic approaches are in need and the cholinesterase inhibitors may be part of combinatorial therapy or serve as a starting point for future drug discoveries.

3.4.1 Synucleinopathy mouse models with cognitive impairment

A great effort has been made towards the generation of relevant models that recapitulate the cardinal features of synucleinopathies. In this respect, given the involvement of αSyn pathology in the course of these diseases, a number of transgenic mouse models overexpressing wild-type (WT) or mutant human αSyn were generated (Kahle, 2008). The major difference between these models is based on the αSyn form that is expressed, either the human WT or mutant proteins, and on the promoter used for the expression. The ultimate goal is that these models recapitulate not only the pathological but also the clinical features of synucleinopathies, and this section will focus on the mouse models that have reported cognitive deficits associated with αSyn pathology.

PDGF-β promoter

The first line described was generated by overexpressing human WT αSyn under the platelet-derived growth factor β (PDGF-β) promoter (Masliah et al., 2000). PDGF-β-αSyn mice presented a predominant pathology in the neocortex, hippocampus and rarely in the substantia nigra, which lead to the characterization of this model as more relevant for DLB than PD (Masliah et al., 2000; Rockenstein et al., 2002). This mouse model showed Morris Water Maze (MWM) deficits only at 9 months of age, indicating spatial learning defects which may arise from an upregulation of the metabotropic glutamate receptor 5 (mGluR5) or from hippocampal neurogenesis inhibition (Masliah et al., 2001; Winner et al., 2004; Price et al., 2010). Specifically, mGluR5 levels were described to be increased in hippocampal and cortical regions in PDGF-β-αSyn mice and brains from PD and DLB patients (Price et al., 2010). Moreover, PDGF-β-αSyn mice showed decreased neurogenesis in the olfactory bulb and hippocampus which was suggested to be mediated by diminished survival of neuronal precursors (Winner et al., 2004). Further studies with PDGF-β-αSyn mouse line reported a link between MWM defects at 12 months of age and a reduction in presynaptic terminal diameter and in postsynaptic density in the temporal cortex (Masliah et al., 2011). Additionally, PDGF-β-αSyn mice showed increased αSyn staining in the neocortex and the

hippocampus (Masliah et al., 2011). These findings strongly support a link between αSyn accumulation in cortex and hippocampal regions as a cause for the cognitive impairment observed in synucleinopathies.

Prion promoter

A mouse line followed in which human αSyn A53T was expressed under the prion promoter, the PrP-αSyn A53T (Giasson et al., 2002). Comparatively with the PDGF-β-αSyn mouse line, the PrP-αSyn A53T mice developed a severer pathology comprising enhanced αSyn aggregation and greater behavioral defects (Giasson et al., 2002). Yet, these defects were mainly characterized by motor symptoms rather than cognitive impairment (Giasson et al., 2002). Interestingly, when they crossed these mice with a transgenic mouse model of AD, the result was a mouse model with significantly increased cognitive impairment when compared with the precursor mouse lines, suggesting a synergistic effect of both pathologies for impaired cognition (Clinton et al., 2010). In contrast, more recent findings reported that the PrP-αSyn A53T mice developed spatial memory deficits in the Y-maze test starting at 6 months of age which were accompanied by synaptic impairment in the hippocampus and progressive aggregation of αSyn (Paumier et al., 2013).

CaMKII promoter

The $Ca2^{+}$/calmodulin-dependent protein kinase II (CaMKII) promoter has also been used to reproduce αSyn pathology mainly in the hippocampus and cortex as it was proposed to lead to high expression levels of the transgene in the forebrain (Nuber et al., 2008). Expression of human WT αSyn under the CaMKII promoter (CaMKII-αSyn mice) resulted in reduced hippocampal neurogenesis at 6 months of age, followed by memory deficits observed by the MWM test at 12 months of age and hippocampal neuron degeneration at 20 months of age (Nuber et al., 2008). Using the same promoter but expressing αSyn A53T (CaMKII-αSyn A53T mice) resulted in contextual fear conditioning deficits, a test that also assesses learning and memory, at 8 months of age which correlated with αSyn aggregation in the hippocampal region (Emre et al., 2007). Increased accumulation of phosphorylated αSyn had foreseen the increased hippocampal and cortical neuron loss, which appeared at 20-22 months of age (Emre et al., 2007). Importantly, these reports demonstrated that cognitive phenotypes precede neuronal loss, implying that memory deficits might arise from the synaptic dysfunction present before neurodegeneration.

Thy1 promoter

Several mouse models using the murine Thy1 neuronal promoter to drive the expression of the transgene have been developed. This promoter has attracted great interest because it is broadly expressed throughout all brain regions and αSyn expression under this promoter does not start until postnatal day 10, thus preventing developmental defects unrelated with human pathology, which occurs at late onset (Chesselet et al., 2012). Focusing on the lines that overexpress mutant versions of αSyn, the expression of human A30P αSyn under the Thy1 promoter (Thy1-aSyn A30P mice) resulted in cognitive impairment at 12 months of age, assayed in the MWM test (Kahle et al., 2000; Freichel et al., 2007). A progressive increase in αSyn pS129 appeared in several brain regions, including the hippocampus, and started at presymptomatic stages. In addition, Thy1-aSyn A30P mice also showed impaired fear conditioning task at 16-19 months of age (Schell et al., 2009). A subsequent report complemented these findings by showing that this mouse model had impaired amygdala and hippocampal synaptic plasticity, given by the progressively lower levels of the synaptic plasticity marker c-Fos and decreased neuronal activity responsive Plk2 (Schell et al., 2012). Other mouse models using the Thy1 driver have been reported, in this case overexpressing non-natural occurring αSyn mutations, namely Y39C and E57K (Zhou et al., 2008; Rockenstein et al., 2014). Extensive transgene expression and αSyn aggregation as well as late onset cognitive impairment were reported in these mice, correlating with extensive pathology in cortical and hippocampal regions (Zhou et al., 2008; Rockenstein et al., 2014). The Thy1 promoter have been used in other mouse models such as the Thy1-aSyn A53T mice, which overexpresses the disease-related A53T αSyn mutant. Although these mice develop several neuropathological features of disease, for instance neuronal loss in several brain regions, mitochondrial dysfunction, motor impairment, among others, no cognitive deficits have been described for this model (Martin et al., 2014; Rothman et al., 2014).

Although αSyn mutations represent a very small percentage of the disease cases, it is unquestionable the critical involvement of αSyn is the vast majority of sporadic cases of the disease. Considering this, a model with the overexpression of the full-length human WT αSyn was developed and extensively characterized, the Thy1-aSyn mice. Contrasting to the previously described models, the Thy1-aSyn mice showed cognitive impairment at early ages resembling the mild cognitive impairment that affects patients at early stages (Rockenstein et al., 2002; Magen et al., 2012). Specifically, at 4-6 months of age, male Thy1-aSyn mice showed deficits in the novel object recognition test (NOR) and in the novel object location (NOL), presented impaired reversal learning in an operant learning task, fewer spontaneous alternations in the Y-maze and learning and spatial memory deficits

assessed by the MWM test (Chesselet et al., 2012; Magen et al., 2012; Ferreira et al., 2017). Additional non-motor impairments were present in these mice such as disturbances in olfactory system, which were described at 3 and 9 months of age and could result from αSyn accumulation in the olfactory bulb (Fleming et al., 2008). Moreover, circadian rhythms were also affected in this line with animals presenting disruptions of the activity/rest cycles around 3-4 months of age and REM sleep dysregulation around 10 months of age (McDowell et al., 2014). Autonomic dysfunction was also developed in these mice (Fleming et al., 2013). Regarding motor impairment, Thy1-aSyn mice showed mild deficits, at 1-3 months of age, comprising impairments in: balance, muscle strength, coordination, vocalizations, fine motor skills and stress-induced defecation (Rabl et al., 2017). In addition, Thy1-aSyn mice showed hyperactivity in a transient period from 4 to 9 months of age, followed by hypoactivity and sensorimotor dysfunction at 15 months of age, where these mice started to exhibit motor symptoms more similar to the ones present in PD patients (Fleming et al., 2006; Lam et al., 2011). With disease progression, mice revealed more difficulties in eating, presented akinesia and developed hunched posture (Chesselet et al., 2012). Neuropathologically, these mice showed widespread αSyn pathology, including proteinase K-resistant αSyn aggregates and increased levels of αSyn pS129 which were progressive and occurred in several brain regions including, but not limited to, the hippocampus, cortex and substantia nigra (Chesselet et al., 2012). Neuronal loss in these animals was reported to occur in the neocortex and some regions of the hippocampus at early stages (3-4 months of age), however, it was not detected dopaminergic neuron loss in the substantia nigra up to 22 months of age (Chesselet et al., 2012; Overk et al., 2014). Nevertheless, dopamine levels were altered throughout time, with an increase of extracellular dopamine levels observed between 6 and 10 months of age and a decrease starting at 14 months of age (Lam et al., 2011). In addition, Thy1-aSyn mice also showed neuroinflammation, which appeared to be progressive and to affect several brain regions, and mitochondrial dysfunction (Rockenstein et al., 2014; Subramaniam et al., 2014; Kim et al., 2015; Price et al., 2018).

Interestingly, the levels of αSyn overexpression in the Thy1-aSyn mice resemble the ones reported for patients with *SNCA* triplication, which consistently develop cognitive impairment and dementia (Chesselet et al., 2012). Considering the described neuropathologic and behavioral features of the Thy1-aSyn mice, this represents a suitable mouse model to study the underlying molecular mechanisms and potential therapeutic approaches targeting cognitive impairment and dementia in LB dementias.

αSyn PFFs injection

Taking into consideration the most recent findings on the field of spreading of αSyn pathology, several mouse models have been developed in this context. One of those models, consisting in the injection of recombinant mouse αSyn PFFs in the muscularis layer of the pylorus and duodenum, resulted in the spreading of αSyn pathology from the gut to the brain *via* the vagus nerve, supporting the Braak hypothesis (Kim et al., 2019). This model recapitulated with some accuracy several features of synucleinopathies, including both motor and non-motor symptoms (Kim et al., 2019). Specifically, at 7 months post injection, dopaminergic neurons degeneration was observed in the substantia nigra and dopamine levels were decreased in the striatum (Kim et al., 2019). At the same age these animals showed impaired motor function and importantly, cognitive decline. This included memory deficits assessed by the Y-maze, NOR and MWM tests and anxiety measured by the open field and elevated plus maze tests. Additionally, these mice also presented social deficits, depression and olfactory and gastrointestinal dysregulation (Kim et al., 2019). Interestingly, vagotomy or *SNCA* deletion prevented the referred neuropathologic and behavioral defects indicating that spreading of pathology is intimately dependent of the presence and seeding of endogenous αSyn protein.

4 Linking alpha-synuclein to the actin cytoskeleton: consequences to neuronal function

Considering the physiologic enrichment of αSyn in presynaptic terminals and its pathological involvement in synucleinopathies, a pathophysiologic interaction of αSyn with elements of the presynapse is expected. One of these elements is the actin cytoskeleton, which plays a key role at the synapse and its dysregulation is being increasingly associated with neurodegeneration. Overall, the impact of αSyn on actin is a critical point to be addressed and the relevance of this interaction for physiology and pathology was reviewed in this thesis (Oliveira da Silva and Liz, 2020).

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Linking Alpha-Synuclein to the Actin Cytoskeleton: Consequences to Neuronal Function

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Alpha-Synuclein (αSyn), a protein highly enriched in neurons where it preferentially localizes at the pre-synapse, has been in the spotlight because its intraneuronal aggregation is a central phenomenon in Parkinson's disease. However, the consequences of αSyn accumulation to neuronal function are not fully understood. Considering the crucial role of actin on synaptic function and the fact that dysregulation of this cytoskeleton component is emerging in neurodegenerative disorders, the impact of αSyn on actin is a critical point to be addressed. In this review we explore the link between αSyn and actin and its significance for physiology and pathology. We discuss the relevance of αSyn-actin interaction for synaptic function and highlight the actin-depolymerizing protein cofilin-1 as a key player on αSyn-induced actin dysfunction in Parkinson's disease.

Keywords: alpha-Synuclein, Parkinson's disease, actin cytoskeleton, actin-binding proteins, cofilin-1

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INTRODUCTION

In the last years, a large body of evidences point to the neuronal cytoskeleton damage as a major contributor to neurodegeneration (Eira et al., 2016). From the various cytoskeleton dysfunctions, alterations in microtubule (MT) stability are a main causative agent in several neurodegenerative disorders and include: (i) variations in the levels of tubulin post-translational modifications with a consequent impact on axonal transport (Dompierre et al., 2007; d'Ydewalle et al., 2011; Zhang et al., 2014; Qu et al., 2017; Magiera et al., 2018), and (ii) dysregulation of MT associated proteins, such as the key example of tau hyperphosphorylation. This modification promotes tau detachment from MTs, what either impacts on axonal transport (Alonso et al., 1997) or leads to recruitment of MT severing enzymes causing axon degeneration (Qiang et al., 2006). Concerning the actin cytoskeleton, dysregulation of this component causing neurodegeneration mostly derives from actin accumulations as the case of cofilin-actin rods (Minamide et al., 2000; Munsie and Truant, 2012).

In the case of Parkinson's disease (PD), the most common synucleinopathy, characterized by the intraneuronal accumulation of aggregated αSyn, several reports demonstrated αSyn-induced alterations of the microtubule cytoskeleton dynamics and axonal transport defects (Carnwath et al., 2018; Prots et al., 2018). As αSyn is a protein that impacts on the synapse both

physiologically and pathologically, an association between the protein and the actin cytoskeleton, a cell component crucial for synaptic function, has been suggested. This review will focus on the current knowledge on the interaction between αSyn and actin and actin-binding proteins (ABPs) concluding with a critical perspective of the implication of these interactions in health and disease. Addressing this topic will certainly contribute with new insights into αSyn-related pathology opening new lines of research targeting neurodegeneration.

ALPHA-SYNUCLEIN: FROM PHYSIOLOGY TO PATHOLOGY

αSyn was originally identified in the *Torpedo* electric organ and is one of the most abundant proteins in the brain (Maroteaux and Scheller, 1991). It belongs to the synuclein family of proteins, a group of small soluble proteins that transiently bind to neuronal membranes, which also includes β-Synuclein and γ-Synuclein (Clayton and George, 1998). αSyn is a 140 amino-acid protein containing three distinct motifs (Maroteaux et al., 1988): the N-terminal contains seven repeats of a 11-residue sequence (XKTKEGVXXXX) responsible for αSyn interaction with vesicles containing phospholipids, the central region contains a non-amyloid component (NAC) sequence which is relatively hydrophobic and prone to aggregation, and the intrinsically unstructured C-terminus region, responsible for multiple protein interactions (Emamzadeh, 2016). αSyn is a natively unfolded protein implying that it lacks a secondary organized structure (Weinreb et al., 1996), what enables the protein to adopt several conformations comprising an unstructured soluble cytosolic form, an α-helical membrane bound structure or a β-sheet-like prone to aggregate conformation (Burre et al., 2013).

αSyn is ubiquitously expressed but highly enriched in the nervous systems (Lavedan, 1998). In neurons, αSyn is enriched in the pre-synaptic terminals where it interacts with synaptic vesicles and participates in several steps of the vesicle cycle comprising trafficking, docking, fusion, and recycling after exocytosis, therefore playing a central role in the regulation of the synaptic transmission (Scott and Roy, 2012; Diao et al., 2013; Wang et al., 2014). Notably, synuclein proteins were proposed to have a major impact in the long-term function of synaptic transmission, as αβγ-Syn triple-knockout mice showed reduced SNARE (SNAP REceptor)-complex assembly, and presented neuropathological signs and shortened lifespan (Burre et al., 2010). Interestingly, although αSyn plays an important role at the pre-synapse, it is one of the last proteins to be targeted to the synapse during development, suggesting that its physiologic function is not focused on the synapse formation but more directed to its maintenance (Cheng et al., 2011). Although αSyn role at the synapse is the most well studied physiological function, a number of additional cellular localizations have been described, including mitochondria, nucleus, endoplasmic reticulum, Golgi complex, and cytoskeleton components, suggesting that the protein contributes to the regulation of innumerous cellular processes (Burre et al., 2018).

αSyn is a protein deeply associated with disease since it was described as the main component of Lewy Bodies (LBs), the pathological hallmark of synucleinopathies (Spillantini et al., 1997), a group of diseases caused by αSyn aggregation and pathology from which PD is the most common one. Although being a cytosolic protein, and its intra-neuronal accumulation resulting in neurodegeneration, it is now known that cell-to-cell transmission of αSyn aggregates also occurs contributing to the progression and propagation of the disease (Karpowicz et al., 2019). Further supporting αSyn link with pathology, familial forms of PD are related with duplication, triplications and point mutations (mainly A30P and A53T mutations) in the *SNCA* gene, encoding for αSyn, which increase the aggregation potential of the protein (Burre et al., 2018).

αSyn aggregated species were shown to induce neurotoxicity through several processes: (i) affecting membrane permeabilization of several cell components, including plasma membrane and endoplasmic reticulum (Colla et al., 2012), mitochondria (Parihar et al., 2009), and vesicle membranes (Volles and Lansbury, 2002; Burre et al., 2018); (ii) increasing reactive oxygen species (ROS) production (Parihar et al., 2009) and Ca^{2+} influx (Danzer et al., 2007); and (iii) disrupting protein synthesis machinery and degradation systems, namely the autophagy-lysosomal and the ubiquitin-proteasomal systems (Lindersson et al., 2004; Garcia-Esparcia et al., 2015). αSyn accumulation was also shown to negatively affect SNARE-complex assembly and disassembly impairing neurotransmitter release and leading to decreased neuronal excitability and synaptic firing, what culminates in synaptotoxicity (Vekrellis et al., 2011). In the same line, αSyn overexpression in neurons decreased spine density and impaired spine dynamics (Blumenstock et al., 2017). Interestingly, synaptic structure and function is highly dependent on actin dynamics, suggesting an impact of αSyn on that cytoskeleton component.

ACTIN CYTOSKELETON: A CRITICAL COMPONENT FOR NEURONAL FUNCTION

The integrity of the cytoskeleton is crucial for neuronal maintenance and function and depends on a critical regulation of its components: actin filaments, microtubules and intermediate filaments. The actin cytoskeleton, the component of interest in this review, is composed of actin filaments (F-actin) that are formed by the association of globular actin (G-actin) to a growing polymer (Mitchison and Cramer, 1996). Actin monomers polymerize/depolymerize from the actin filament constituting the fundamental process driving actin dynamics (Carlier, 1998). There is a considerable number of ABPs regulating actin dynamics among which are: (i) nucleation factors (formins and Arp2/3) that promote the assembly of G-actin into filaments and the development of branched networks (Bugyi et al., 2006; Korobova and Svitkina, 2008); (ii) actin-monomer binding proteins (profilin) that provide new subunits to the filament enhancing the assembly of G-actin into F-actin (Mockrin and Korn, 1980); (iii) proteins that bundle (fascin)

or crosslink (α-actinins and filamins) the actin filaments (Tseng et al., 2004); (iv) tropomyosins which are able to regulate actin dynamics through binding along F-actin and interaction with other ABPs or by direct contact of F-actin with different tropomyosin isoforms (Gunning et al., 2008; Schevzov et al., 2012); (v) spectrins that are localized at subcortical regions linking actin cytoskeletal meshwork to membrane receptors (Burridge et al., 1982); (vi) capping proteins (adducin) which bind to actin filaments blocking their growth (Matsuoka et al., 2000) and (vii) severing proteins (actin depolymerizing factor (ADF)/cofilin-1 and gelsolin) which control the rate of actin polymerization (Weeds et al., 1991; Andrianantoandro and Pollard, 2006).

In neurons, actin acquires several structural rearrangements that are differentially distributed in the cell. Lamellipodia is an actin structure characterized by a branched network of short actin filaments while filopodia is composed of long parallel bundles of actin filaments (Neukirchen and Bradke, 2011). Lamellipodia and filopodia are actin arrangements enriched in the more dynamic neuronal structures including the growth cone, dendrites and dendritic spines. In the growth cones, besides filopodia and lamellipodia, there is, in the transition zone, an actomyosin contractile structure named actin arcs which are perpendicular to F-actin and are suggested to interact with microtubules and allow them to invade the growth cone (Schaefer et al., 2002). In dendritic spines, actin is the core structure providing the architecture for the formation, stability, motility, and morphology of the spines (Basu and Lamprecht, 2018). Actin patches are structurally similar to lamellipodia and are found in the axons and dendrites (Korobova and Svitkina, 2010; Spillane et al., 2011). It is suggested that they promote locally actin filopodia formation, as these actin patches are not motile structures (Spillane et al., 2011). More recently, a component of the neuronal subcortical cytoskeleton was described, the actin rings (Xu et al., 2013). This structure is along the axon and dendrites and is composed by a ring of short actin filaments capped by adducin and distanced by ∼190 nm by spectrin (Xu et al., 2013). More recently, non-muscle myosin II was proposed to regulate the axonal actin ring contraction and expansion (Costa et al., 2020). While actin rings are considered to give mechanical support to neurons, there is a dynamic pool of actin in the structure of the axon, termed actin trails, which have been suggested to be composed of actin "hotspots" and provide for a flexible actin cytoskeleton network in the axon (Ganguly et al., 2015).

In mature neurons a dysregulation of the actin cytoskeleton has tremendous implications for spine density, morphology and function (Zhang and Benson, 2001). An emergent number of studies have also reported the abundance and relevance of actin and ABPs in the pre-synaptic terminals, where actin dynamics plays essential functions on the vesicle pool organization, synaptic vesicle mobilization and exocytosis and posterior endocytosis (Rust and Maritzen, 2015). The crucial roles of actin at the synapse turns it an important protein to study in the context of neurodegenerative disorders where synaptic dysfunction is a central causing agent.

αSYN-ACTIN CYTOSKELETON LINK: IMPACT ON NEURONAL HEALTH AND DISEASE

Evidences of αSyn Interaction With Actin and Actin-Binding Proteins

The most αSyn recognized physiological function is on the synaptic vesicle cycle, a process where actin plays a key role. This functional "proximity" between αSyn and actin boosted the research on identifying an interaction between the two proteins. This interaction was investigated mainly in *in vitro* assays, either with cell-free or with cell-line approaches, which although were critical for the demonstration of the interaction of αSyn with actin and actin-binding proteins (Zhou et al., 2004; Peng et al., 2005; Esposito et al., 2007; Sousa et al., 2009; Welander et al., 2011; Lee et al., 2012), lack the validation in primary neuronal cultures which would be a more relevant scenario. In this respect, in a cellular PD model using rotenone-treated dopaminergic neurons, it was observed an increase in the expression levels of F-actin and αSyn. However, a putative interaction between the two proteins was not explored in that context (Mattii et al., 2019). Further supporting an interaction, αSyn and actin co-immunoprecipitated in rat brain homogenates under physiological conditions (Sousa et al., 2009).

Additionally, proteomic studies demonstrated alterations on the expression levels of several ABPs in models of αSyn overexpression in *D. melanogaster* (Xun et al., 2007a,b) and *C. elegans* (Ichibangase et al., 2008). Moreover, gelsolin was found in LBs from PD and Dementia with Lewy Bodies (DLB) patients and was shown to have a positive effect on αSyn aggregation in the presence of high Ca^{2+} concentrations (Welander et al., 2011).

αSyn Impact on Actin Dynamics: Cofilin-1 Involvement

Based on the data demonstrating a putative interaction between αSyn and actin, it was investigated whether αSyn directly binds to actin modulating actin dynamics. *In vitro* cell-free assays showed that WT αSyn decreased the rate of polymerized actin, an effect suggested to occur due to the αSyn-mediated sequestration of the actin monomers. Interestingly, this effect was decreased in the presence of high concentrations of Ca^{2+}, a scenario mimicking a stimulated state of neurons. In opposite, A30P aSyn, increased actin polymerization and stabilization of the actin filaments (Sousa et al., 2009). Validation of the impact of αSyn on actin dynamics was performed in studies with neuronal cell lines and primary cultures of hippocampal neurons, expressing either WT or A30P αSyn, which demonstrated that physiologically WT αSyn regulates actin dynamics, while the pathologic A30P aSyn disrupts the actin cytoskeleton (Sousa et al., 2009).

The proposed physiologic impact of WT αSyn on actin dynamics, and the fact that both αSyn and actin play a role on the synapse, raise the question of whether αSyn-actin interaction might regulate neurotransmitter homeostasis. Supporting this hypothesis, a report demonstrated a critical dependence of the interaction of αSyn with the actin cytoskeleton for the

trafficking and transport activity of norepineprhine transporters (Jeannotte and Sidhu, 2008).

Concerning the pathological impact of αSyn on the actin cytoskeleton, further studies showed that the extracellular addition of high concentrations of WT or A30P αSyn to hippocampal neurons induced a stabilization of the actin cytoskeleton, by increasing the number of lamellipodia and filopodia and resistance to depolymerization, with the mutant protein having a more pronounced effect. These αSyn-induced actin alterations were mediated by the activation of the actin signaling pathway Rac1/PAK2/LIMK/cofilin-1 and to require GRP78, an endoplasmic reticulum chaperone present at the cell membrane of several cell types (Bellani et al., 2014). Cofilin-1 is an actin depolymerizing protein which activity is negatively regulated by phosphorylation in the Serine 3 residue, promoted by several kinases, including LIMK (Arber et al., 1998). As such, pathologic αSyn induced cofilin-1 phosphorylation leading to inactivation of its depolymerizing action and consequently to actin stabilization. Importantly, this outcome was recapitulated using fibroblasts from PD patients carrying multiplications of the *SNCA* gene (Bellani et al., 2014), which presented a threefold increase in the phospho-cofilin 1/cofilin 1 ratio and an increased number and thickness of actin stress fibers structures (Bellani et al., 2014).

Additional work with primary hippocampal neurons confirmed a negative effect of pathologic αSyn on actin dynamics, mediated by cofilin-1 inactivation, which impacted on neuronal functions such as axon elongation and migration (Tilve et al., 2015). Additionally, and also supporting αSyn-induced cofilin-1 inactivation, in a glaucoma animal model, consisting on elevated intraocular pressure which results in retinal neurodegeneration, the intravitreal injection of αSyn antibodies hampered neurodegeneration, an effect that was suggested to involve upregulation of cofilin-1 (Teister et al., 2017).

The presented studies claim that pathologic concentrations of αSyn induce cofilin-1 inactivation resulting on actin stabilization and neuronal dysfunction. However, cofilin-1 was also placed in the context of αSyn-induced neurodegeneration in a different scenario. In a study addressing the mechanisms of protein aggregates entry in cells, downregulation of cofilin-1 decreased αSyn aggregates entry, while both, the silencing of ROCK1 and the pharmacological inhibition of Rho, increased aggregate entry (Zhong et al., 2018). These observations suggested Rho-ROCK1-LIMK-Cofilin-1 pathway as a relevant signaling cascade triggering αSyn aggregates entry in the host cells (Zhong et al., 2018). Regardless of the context, it is remarkable the impact of αSyn in the actin cytoskeleton through cofilin-1.

αSyn-Induced Disruption of the Actin Cytoskeleton in *in vivo* Models of PD

In the previous sections the analysis of the effect of αSyn on the actin cytoskeleton was mainly derived from cell-based studies. It is important to understand what happens *in vivo* by using disease models. In this respect, a report using a PD *Drosophila* model based on the neuronal overexpression of αSyn, validated the pathologic impact of the protein on the actin cytoskeleton, as αSyn transgenic flies showed increased F-actin and the presence of rod-shaped actin-cofilin rich inclusions in whole-mounted brains (Ordonez et al., 2018). Rod structures were also observed in the brainstem region of a PD mouse model expressing the A53T mutant form of αSyn, and in the cingulate cortex region of a DLB patient (Ordonez et al., 2018). Following experiments in fly demonstrated that the disruption of the actin cytoskeleton induced by αSyn was mediated by its interaction with α-spectrin, and resulted on the mislocalization of the mitochondrial fission protein Drp1 and subsequent mitochondrial dysfunction (Ordonez et al., 2018). This study pointed to a critical interaction between αSyn and α-spectrin resulting in actin dysfunction which consequently affects mitochondria. Additionally, and although not highly explored, the study also shows the scenario of αSyn induction of cofilin-actin rods. These are structures formed upon localized cofilin-1 activation (by dephosphorylation), leading to its association to F-actin and promoting the formation of short actin filaments saturated with cofilin-1. Rod formation has been mainly studied in the context of Alzheimer's disease (AD) and shown to have a tremendous impact in neurons causing synaptic dysfunction, blocking axonal transport, and exacerbating mitochondrial membrane potential loss what culminates in cognitive impairment (Bamburg and Bernstein, 2016). Considering the impact of cofilin-actin rods on neurodegeneration, the pathologic relevance of rod formation upon αSyn overexpression should be further addressed. In this respect, one study reported the presence of oxidized γ-Synuclein in cofilin-actin rods in the thalamus of mice subjected to traumatic brain injury (Surgucheva et al., 2014).

DISCUSSION

The current revision summarizes the studies supporting the link between αSyn and the actin cytoskeleton. This is an important topic as while the interplay between αSyn and the microtubules was recently reviewed (Carnwath et al., 2018; Calogero et al., 2019), the link αSyn-actin was less explored. Considering the αSyn structural features responsible for the interaction with cytoskeleton components, it was described that αSyn is a functional microtubule-associated protein with its C-terminal region suggested to be responsible for the interaction with microtubules (Alim et al., 2004; Cartelli et al., 2016). In the case of αSyn interaction with actin, although it is implied by the available literature, no structural features underlying this interaction have been explored to date what deserves future investigation.

One important question raised by this review is whether physiologically αSyn, by regulating actin dynamics, impacts on the actin-derived functions on synaptic transmission. In this respect, while there are studies with αSyn KO mice showing decreased SNARE-complex assembly and changes in synaptic structure and size (Burre et al., 2010; Greten-Harrison et al., 2010), other reports showed no major defects in synaptic function in αSyn KO mice (Abeliovich et al., 2000; Chandra et al., 2004; Chadchankar and Yavich, 2011). Taking this into consideration,

it would be important to explore neuronal actin dynamics and actin organization in synaptic structures in αSyn KO mice. This would clarify whether the αSyn-actin link acts on the physiology of the synapse, what could explain the impairment of synaptic activity in PD.

The most striking point of the present review is the highlight of cofilin-1 as a central player in αSyn-induced neuronal dysfunction. Cofilin-1 depolymerizing activity upon actin is crucial for synaptic function since the remodeling of the pre- and post-synapses intimately relies on actin dynamics (Pontrello et al., 2012). As such, we might hypothesize that the reported αSyn-activation of the actin signaling pathway Rac1/PAK2/LIMK/cofilin-1 via GRP78 (Bellani et al., 2014) which results on cofilin inactivation, and consequent blockage of actin dynamics (Sousa et al., 2009; Bellani et al., 2014) occurs at the pre-synapse, and might contribute for the synaptic dysfunction observed in the several synucleinopathies. An additional pathway by which pathogenic αSyn might cause synaptic dysfunction is *via* the induction of cofilin-actin rods formation (Cichon et al., 2012), which was observed in a PD *Drosophila* model (Ordonez et al., 2018). In AD, Aβ-induced formation of rods occurs through a pathway involving the cellular prion protein (PrP^C) and NADPH oxidase (NOX) (Walsh et al., 2014), which results in the dysregulation of cofilin activity *via* oxidation and dephosphorylation, and consequent formation of cofilin-actin rods. Interestingly, αSyn was shown to interact with PrP^C to induce synaptic dysfunction (Ferreira et al., 2017). This finding raises the question of whether, similarly to what occurs in AD, αSyn-induced rod formation is mediated through a PrP^C-dependent pathway culminating on disruption of synaptic activity.

While the literature suggests that αSyn might impact on neuronal function, through modulation of cofilin-1 activity, is still unclear whether αSyn induces an activation or inactivation of the ABP. Interestingly, a similar scenario was seen in AD where cofilin-1 activity was shown to be regulated in multiple ways depending on the pathogenesis context (Kang and Woo, 2019). Importantly, inhibition of cofilin activity or expression was shown to have ameliorative effects in AD (Deng et al., 2016). In the case of αSyn-induced neurodegeneration, it will be important to analyze the phosphorylation status of cofilin-1 in different pathological scenarios and cellular contexts, as well as the impact of cofilin-actin rods formation for neuronal function. These studies will be critical to point cofilin-1 as novel therapeutic target to prevent neurodegeneration in synucleinopathies.

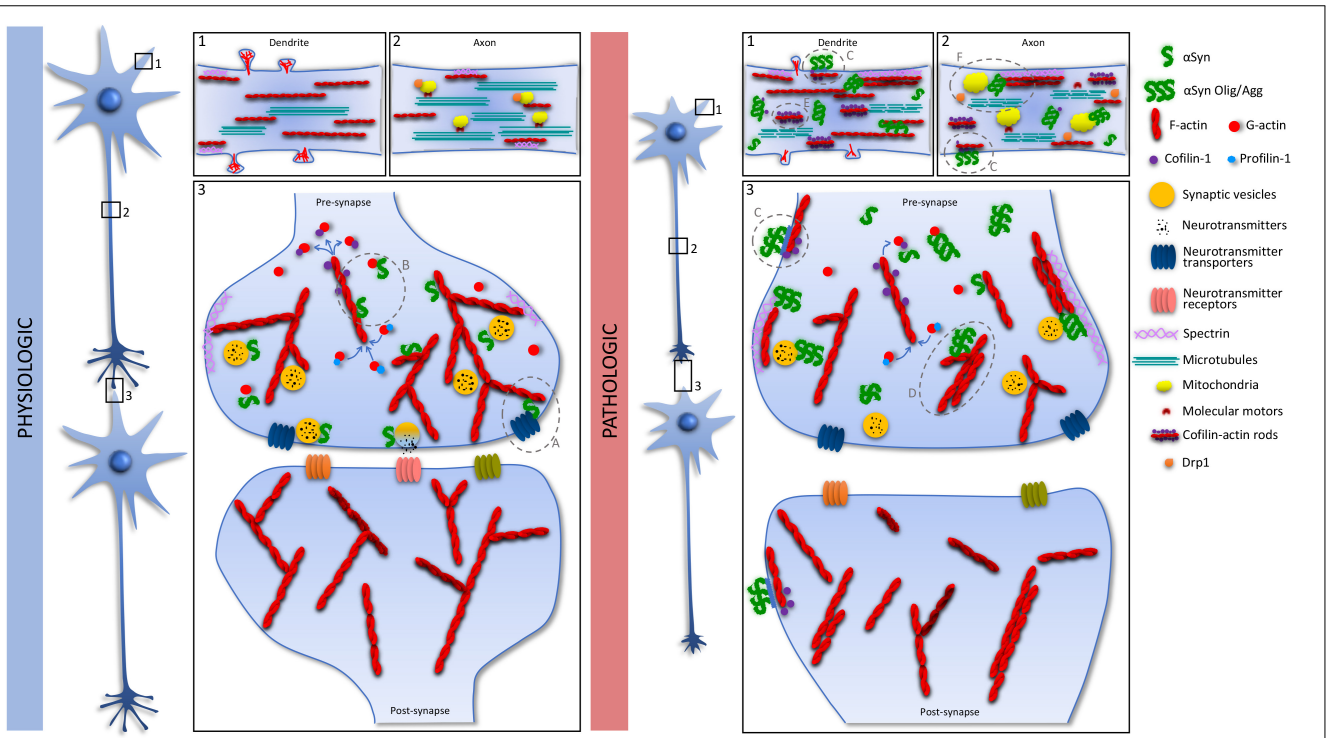

FIGURE 1 | Schematic representation of the link between αSyn and actin. Under physiologic conditions αSyn is mainly concentrated at the pre-synapse and interacts with synaptic vesicles at the several stages of the vesicle cycle. At the pre-synapse αSyn-actin interaction was described as being crucial for the trafficking and transport activity of neurotransmitter transporters (Physiologic, Panel 3; **A**). Additionally, we suggest that the reported αSyn regulation of actin dynamics might contribute for the proper actin-derived functions at the pre-synapse (Physiologic, Panel 3; **B**). Under pathologic conditions, overexpression and misfolding of αSyn affects several cellular processes. At the cell membrane, extracellular misfolded αSyn uses a pathway culminating in cofilin-1 activation and actin remodeling to enter the cells (Pathologic, Panels 1–3; **C**). Intracellularly, synaptic dysfunction might occur due to the αSyn-induced stabilization of the actin cytoskeleton (Pathologic, Panel 3; **D**), through inactivation of cofilin-1 or αSyn-induced cofilin-actin rod formation (Pathologic, Panel 1; **E**), that also affects axonal transport. Additionally, αSyn interaction with spectrin (Pathologic, Panel 2; **F**), disrupts the actin cytoskeleton with consequent mislocalization of Drp1 and mitochondrial impairment.

Another point that stands out from the studies reported in this review is that the few reported experiments in primary neurons were performed with cultures of hippocampal neurons (Sousa et al., 2009; Bellani et al., 2014; Tilve et al., 2015). Although this observation suggests that additional studies should be performed with primary cultures of dopaminergic neurons, the cell type mainly affected in typical motor PD, research on the impact of αSyn on actin in hippocampal neurons is also essential considering the symptomology of dementia which has been linked to Syn pathology in the hippocampus leading to neuronal dysfunction (Hall et al., 2014).

In summary, this review presents a critical perspective in the αSyn impact on the actin cytoskeleton. The literature here revised strongly suggests that αSyn interacts/modulates actin and ABPs what has consequences to pathophysiology, as summarized in **Figure 1**. Nevertheless, this topic requires further investigation what might be of extremely importance in the context of both health and disease.

AUTHOR CONTRIBUTIONS

MO and ML conceived the structure and content and wrote the manuscript. MO produced the figure. Both authors contributed to the article and approved the submitted version.

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RESEARCH AIMS

The main goal of this Thesis was to determine if hippocampal cofilin pathology constitutes a driver of synaptic dysfunction and cognitive impairment in Lewy Body dementias. To address this we proposed the following objectives:

- Understand the impact of αSyn overexpression on cofilin pathology, including cofilin activation and cofilin-actin rods formation, *in vitro* using primary cultures of hippocampal neurons, and *in vivo* using the Thy1-aSyn mice (Chapter I).

- Dissect the molecular mechanisms and the functional consequences of αSyn overexpression-induced cofilin dysregulation in hippocampal neurons (Chapter I).

- Explore the effect of exogenous αSyn and of αSyn spreading and seeding in cofilin-actin rod formation *in vitro*, in hippocampal neurons, and *in vivo,* in a mouse model of αSyn pre-formed fibrils (αSyn PFFs) injection (Chapter II).

PROLOGUE

Dissecting the impact of amyloidogenic TTR in the neuronal cytoskeleton using a fly model

My interest in studying actin cytoskeleton alterations in neurodegenerative diseases started with an initial project in which I studied the impact of the amyloidogenic protein transthyretin (TTR) in the neuronal cytoskeleton using *Drosophila melanogaster* as a model.

Background

Familial Amyloid Polyneuropathy (FAP) is a neurodegenerative disease characterized by the deposition of mutated transthyretin (TTR) in the form of amyloid fibrils, particularly in the peripheral nervous system. The most common pathogenic substitution is Val30Met and as a consequence of TTR deposition axonal degeneration occurs, resulting in neuronal death with disease progression. Despite the intensive research performed in the field, the mechanisms underlying the molecular pathways by which TTR causes neurodegeneration are still unclear. In this work we aimed at characterizing the cytoskeleton alterations underlying the TTR-induced neurodegeneration as well as identifying modulators of this alteration through a small-guided genetic screen using as candidates the major regulators of the cytoskeleton. To achieve this, we used a *Drosophila* model overexpressing TTRV30M in the photoreceptor cells.

During my PhD this research originated a publication in *Scientific Reports* which constitutes the prologue of this thesis:

Oliveira da Silva, M. I., Lopes, C. S., & Liz, M. A. (2020). Transthyretin interacts with actin regulators in a Drosophila model of familial amyloid polyneuropathy. *Scientific reports*, *10*(1), 1-9.

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Transthyretin interacts with actin regulators in a *Drosophila* model of familial amyloid polyneuropathy

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Familial amyloid polyneuropathy (FAP) is a neurodegenerative disorder whose major hallmark is the deposition of mutated transthyretin (TTR) in the form of amyloid fibrils in the peripheral nervous system (PNS). The exposure of PNS axons to extracellular TTR deposits leads to an axonopathy that culminates in neuronal death. However, the molecular mechanisms underlying TTR-induced neurodegeneration are still unclear, despite the extensive studies in vertebrate models. In this work we used a *Drosophila* FAP model, based on the expression of the amyloidogenic TTR (V30M) in the fly retina, to uncover genetic interactions with cytoskeleton regulators. We show that TTR interacts with actin regulators and induces cytoskeleton alterations, leading to axonal defects. Moreover, our study pinpoints an interaction between TTRV30M and members of Rho GTPase signaling pathways, the major actin regulators. Based on these findings we propose that actin cytoskeleton alterations may mediate the axonopathy observed in FAP patients, and highlight a molecular pathway, mediated by Rho GTPases, underlying TTR-induced neurodegeneration. We expect this work to prompt novel studies and approaches towards FAP therapy.

Familial amyloid polyneuropathy (FAP) is a fatal autosomal dominant disease characterized by the extracellular deposition of amyloid fibrils of mutated transthyretin (TTR), particularly in the peripheral nervous system (PNS)[1,2]. As a consequence of TTR deposition, a dying-back axonopathy develops which is followed by neuronal degeneration in advanced disease stages[3]. Physiologically, TTR is mainly synthesized in the liver and choroid plexus of the brain, and secreted to the blood and cerebrospinal fluid (CSF)[4]. TTR expression is also found in the retinal pigment of the eye and in pancreas[5,6]. TTR most acknowledged functions are the transport of thyroxine and retinol[7]. However, it is also an important player in nerve biology being able to increase axon regeneration after sciatic nerve crush and to promote neurite outgrowth of PNS neurons[8].

More than one hundred TTR variants have been reported, being TTRV30M the most frequent mutation associated to FAP. TTRV30M patients develop a progressive peripheral neuropathy usually accompanied by autonomic dysfunction, although TTR amyloid deposits are also found in the eye, kidney, heart and gastrointestinal tract[3]. Central nervous system (CNS) deposits, although rare, have also been reported[9].

How TTR extracellular aggregates induce intracellular events in FAP is not yet fully understood. Although the dying-back axonal degeneration might suggest a disturbance of the distal cytoskeleton, as a consequence of TTR deposition, cytoskeleton alterations, as reported in other neurodegenerative disorders[10], were not previously addressed. Nevertheless, pathways previously described as mediators of TTR neurotoxicity, such as calcium influx and the binding to the receptor for advanced glycation endproducts (RAGE), might signal actin cytoskeleton-related pathways[11–13].

In this work we aimed to characterize actin cytoskeleton-related pathways involved in FAP-associated axonopathy. We used a FAP *Drosophila* model, expressing human TTRV30M in the fly retina, under the control of GMR promoter, that recapitulates FAP features, namely protein aggregation, similarly to what was previously reported in flies expressing TTRV30M under the pan neuronal *elav* promoter[14]. Using this model, we characterized a novel genetic interaction between TTRV30M and members of Rho GTPase-regulated pathways, major players in actin dynamics and implicated in several neurodegenerative disorders[15]. Considering the high homology and

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functional conservation observed between the fly and vertebrate neuronal cytoskeleton[16] we believe that this study can provide valuable insights into the molecular basis of FAP.

Results

Characterization of a FAP fly model based on the expression of amyloidogenic TTR in the retina. Previous studies showed that ectopic expression of TTR amyloidogenic mutant isoforms in *Drosophila* induced neurodegeneration[14,17]. In particular, expression of TTRV30M in the developing photoreceptors induces a rough eye phenotype in the adult fly[14]. We used this observation as a starting point to our study, which aimed to analyse whether actin regulators could act as modifiers of the rough eye phenotype of TTRV30M expressing flies. Our analysis showed that flies expressing WT TTR, under the control of GMR-Gal4, had normal size retinas without visible morphological alterations, similar to control flies expressing GFP, as revealed by scanning electron microscopy (Fig. 1A,B). In contrast, ectopic expression of TTRV30M induced smaller eyes, with a severe rough phenotype, characterized by fused and disordered ommatidia, lack of mechanosensory bristles and with foci of necrotic lesions (Fig. 1C). These results illustrate the specific impact of amyloidogenic TTRV30M. We confirmed TTR expression by western blot analysis and observed no differences in TTR protein levels between GMR>TTR^{V30M} and GMR>*TTR* flies, discarding the hypothesis that the phenotype observed results from differential expression levels between TTR isoforms (Supplementary Fig. S1A). Next we evaluated the TTR expression pattern in the developing eye-imaginal disc, by comparing with GFP expression within the same genetic background (GMR>*GFP;*TTR^{V30M}). Our analysis showed that while GFP expression was restricted to the posterior domain of the developing eye, TTRV30M was detected both in the posterior and anterior domains (Supplementary Fig. S1B,C). These results demonstrate that TTR is secreted to the adjacent tissues (Supplementary Fig. S1C), despite expression driven by GMR promoter being limited to the posterior domain of the eye imaginal disc. Our results are in agreement with data obtained with other TTR-amyloidosis fly models, where TTR was shown to be secreted from photoreceptors and to circulate to other tissues[17].

Previous studies modeling FAP in *Drosophila* showed that expression of mutant forms of TTR reduced lifespan and impaired climbing ability, signs of neurologic impairment in flies[17]. We recapitulated these analyses using GMR promoter to drive expression of TTRV30M. Our results show that GMR>TTR^{V30M} flies have a shorter lifespan when compared to control flies (GMR>*GFP*) (Fig. 1D). The median survival rate of GMR>TTR^{V30M} flies was reduced by 35% (from 37 to 24 days) relative to control flies (GMR>*GFP*). In contrast, flies expressing wild-type TTR presented no marked alterations in lifespan having a median survival of 33 days (Fig. 1D). We next evaluated the climbing activity of both genetic backgrounds (Fig. 1E). The climbing ability of control flies was maintained during the course of the experiment, however the climbing ability of TTRV30M expressing flies dramatically decreased to 40% and 22%, by days 10 and 15, respectively. This clearly shows that the phenotype is aggravated with aging. For the same time points, the climbing ability of TTR expressing flies decreased to 67% and 35%, revealing a less accentuated effect of the wild-type TTR on neuronal functions (Fig. 1E).

Following the characterization of the phenotype of the GMR>TTR^{V30M} flies, which revealed rough eye phenotype at day 1 and behavior alterations being aggravated with aging, we confirmed protein aggregation in our model. Using western blot analysis of soluble and insoluble fractions of head extracts from GMR>TTR^{V30M} flies, we observed the presence of insoluble TTRV30M aggregates in 1 and 15-days old flies, which were increased with age (Fig. 1F). In TTRV30M larval brains no insoluble aggregates were detected (Fig. 1F). However, considering the amount of aggregated protein at day 1, we cannot exclude the formation of soluble toxic oligomers at this stage.

TTRV30M-associated neurotoxicity is also detected when expression is limited to adult stages. Using the $Gal80^{ts}$ system we promoted TTRV30M expression in the retina during adult stage, and assayed flies climbing performance. Our analysis shows that TTRV30M expressing flies show increased climbing defects by day 20, relative to controls (Fig. 1G). Altogether, these findings highlight the specific neurotoxicity of TTRV30M and validate the use of GMR>TTR^{V30M} flies as a relevant model to use in genetic screens aiming at identifying modifiers of TTRV30M induced- phenotype.

TTRV30M induces actin cytoskeleton alterations in photoreceptor axons. As in many other studies using *Drosophila* to model neurodegeneration, we found that TTRV30M expressing flies have a severe rough eye phenotype by day 1. Actually, this suggests that amyloidogenic TTR might have an impact on neuronal morphogenesis. As such, we evaluated the cytoskeleton organization of photoreceptor axons at the third instar larval stage by crossing GMR>TTR^{V30M} flies with Lifeact expressing flies, to label F-actin. We observed that contrarily to control flies (Fig. 2A), TTRV30M expressing larvae exhibited defects in axonal projections. In 8% of the TTRV30M expressing larvae axons failed to project into the brain area although photoreceptors differentiated within the eye disc (data not shown); in the remaining 92%, axons extended projections into the brain region, however the majority (66.7%) in a highly disorganized manner (Fig. 2B,C). The abnormal projections observed in TTRV30M expressing larvae comprised axons with misguided orientation, with some of them failing to reach the proper target layer and apparently growing in an opposite direction. Within the medulla, axons also projected in an irregular manner, failing to reach their target, and some medulla regions lacked axonal projections (Fig. 2B). In addition, growth cones of projecting axons lacked the normal spread distribution of filopodia and lamellipodia actin structure, suggesting altered actin organization (Fig. 2B′).

Additionally, we analyzed axonal projections of PDF expressing neurons in GMR>TTR^{V30M} flies (Supplementary Fig. S2). Abnormal axonal arborization was detected in larvae, that was clearly aggravated in adult stage (Supplementary Fig. S2C,D). The area of PDF expressing neurons is larger in GMR>TTR^{V30M} adult flies (Supplementary Fig. S2D,E). We also observed a disorganized spatial arrangement of cell bodies of PDF expressing

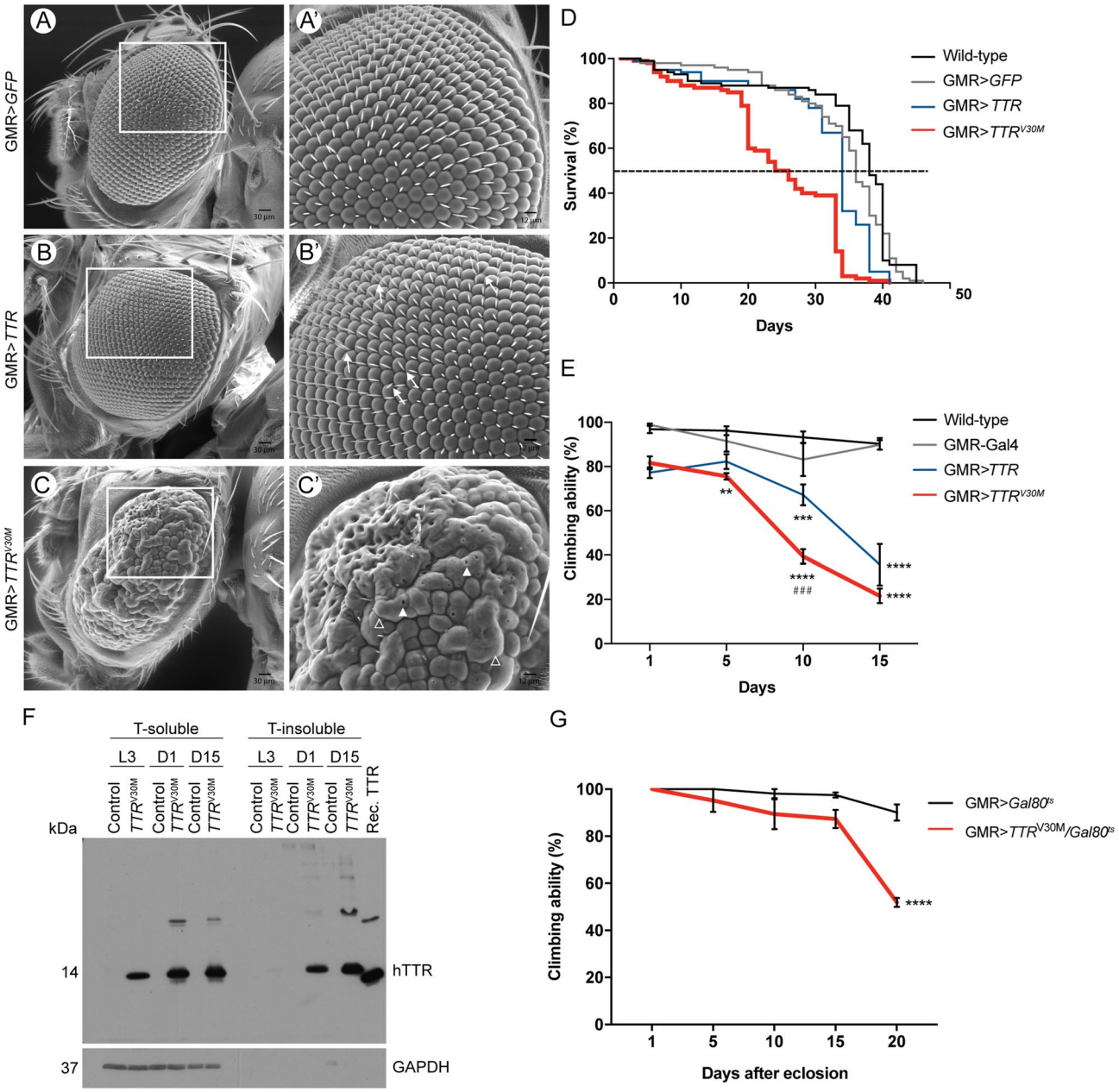

Figure 1. TTRV30M induces neurodegeneration in *Drosophila*. (**A**–**C**) Representative images of scanning electron microscopy (SEM) of fly retinas of the indicated genotypes. (**A**, **A′**) Control flies (GMR>*GFP*) show normal eyes with regular arrangement of ommatidia and bristle orientation. (**B**, **B′**) Flies expressing wild-type TTR display mild irregularities comprising loss or misorientation of bristles (**B′**, white arrows). (**C**, **C′**) Flies overexpressing TTRV30M have smaller eyes, fewer bristles and fused ommatidia (**C′**, white open arrowheads); small holes can be found in the surface of the ommatidia (**C′**, white arrowheads). Scale bar: (**A**–**C**) = 30 µm; (**A′**–**C′**) = 12 µm. (**D**) Lifespan analysis of the genotypes under study. The dashed line indicates the day at which 50% of the flies die. n ≥ 100 flies per genotype. (**E**) Climbing ability of the indicated genotypes. Tests were performed every 5 days during 15 days, after hatching. n = 60 flies per genotype; the results represent the mean ± SEM. Statistical significance determined by Two-Way ANOVA with Turkey's multiple comparisons test: asterisks (*) significance relative to control flies; cardinals (#) significance of GMR>TTR^{V30M} relative to GMR>TTR^{WT}; ***p < 0.001, ###p < 0.001, ****p < 0.0001. (**F**) Western blot analysis of soluble/insoluble TTR in fly extracts. Control (GMR-G4) and GMR>TTR^{V30M} flies were analyzed for the presence of TTR in Triton-soluble (T-soluble) and Triton-insoluble (T-insoluble) fractions of the protein extracts. Extracts from larval stages (L3), 1-day old flies (D1) and 15-day old flies (D15) were analyzed. Immunoblot for TTR confirmed the presence of the protein in soluble fractions from larvae and adult flies, and also the presence of TTR in the insoluble fractions of 1- and 15-day old flies. Recombinant TTR (Rec. TTR) was used as positive control. Immunoblot for GAPDH was used as a loading control, confirming the presence of the soluble proteins in the T-soluble fractions. (**G**) Climbing ability of the indicated genotypes. Tests were performed every 5 days during 20 days, after hatching. n ≥ 65 flies per genotype; the results represent the mean ± SEM. Statistical significance determined by Two-Way ANOVA with Sidak's multiple comparisons test.

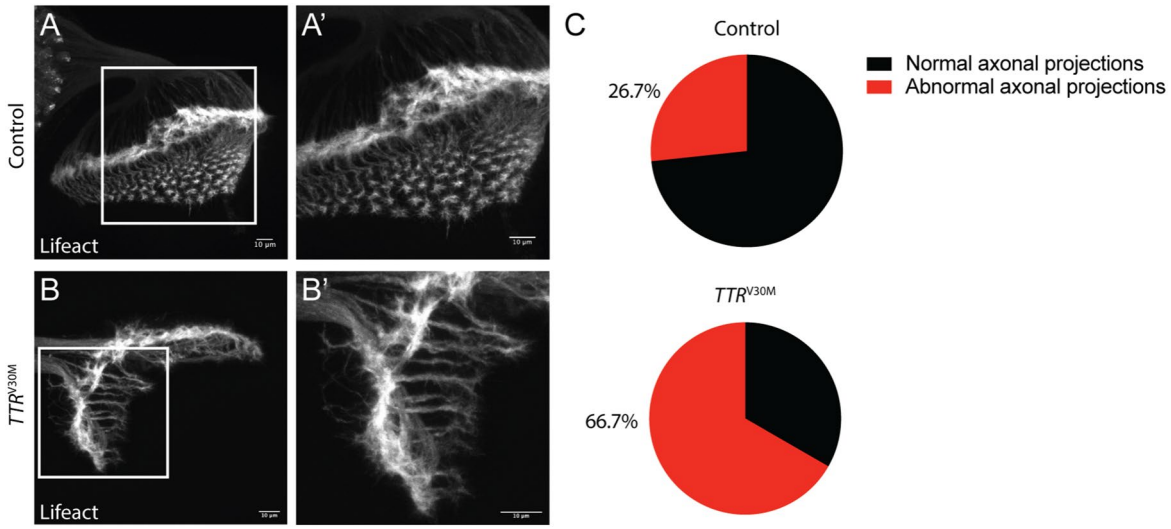

Figure 2. TTRV30M expression in the retina induces axonal projections defects. (**A**, **B**) The actin reporter UAS-Lifeact-ruby was used to evaluate axonal projections from 3rd instar eye-imaginal discs into larval brains. (**A**, **A′**) In GMR>*Lifeact* larvae (control) retinal axons project into the lamina and medulla layers in an organized manner. (**A′**) High magnification of (**A**) showing the normal array of the axonal projections and growth cones in the medulla layer. (**B**, **B′**) GMR>*Lifeact;TTR*^{V30M} expressing larvae (*TTR*^{V30M}) exhibit abnormal axonal projections. (**B′**) High magnification of (**B**) showing a disruption of the typical star-shaped structure of the growth cones. Scale bar: 10 µm. (**C**) Pie chart representation of the percentage of eye imaginal discs that display axonal abnormalities, as determined by qualitative analysis. GMR>*Lifeact* (Control, n = 15); GMR>*Lifeact;TTR* ^{V30M} (*TTR*^{V30M}, n = 12).

neurons (Supplementary Fig. S2D′). These results indicate that TTRV30M toxicity influences the cytoskeleton, impacting on axonal projection and organization.

TTR interacts genetically with members of Rho GTPase signaling pathways. The retina phenotype associated with TTRV30M expression was the basis for a genetic screen aimed to identify actin cytoskeleton regulators that might represent novel TTR interacting molecules. We evaluated TTR interactions using null alleles for each gene under test. The use of mutant alleles has the advantage of avoiding the fluctuating levels of RNAi-mediated downregulation and possible off-target effects. For control, GMR>*TTR*^{V30M} homozygous flies were crossed with wild-type flies so that there is only one copy of the UAS-*TTR*^{V30M} transgene in the background, similar to what is obtained after crossing GMR>*TTR*^{V30M} with the different mutant alleles. Heterozygous flies for mutations in each of these genes do not show eye phenotype. Our screen was focused on members of the Rho GTPase family, major regulators of actin dynamics and implicated in several neurodegenerative disorders[15]. We classified as suppressors or enhancers the interactions leading to a reduction or increase in the percentage of flies with severe rough eye phenotype (Fig. 3A), respectively. We observed that haploinsufficiency for *rho1* and *rok* induced an increase in the percentage of flies with severe rough eye phenotype (Fig. 3B). These results imply that downregulation of the Rho1-Rok pathway acts as an enhancer of TTRV30M-induced neurotoxicity. Haploinsufficiency for *rac1/2* and *cdc42* induced a suppression of the rough eye phenotype. In agreement with these observations, decreasing the levels of Pak and LIMK, downstream effectors of Rac1 and Cdc42, also suppressed the TTRV30M-induced phenotype (Fig. 3B). Additionally, haploinsufficiency for *twinstar* (*tsr*), the *Drosophila* homolog for cofilin/actin depolymerization factor (ADF), and *chickadee* (*chic*), the *Drosophila* homolog of profilin, two actin binding proteins, crucial for actin dynamics regulated by Rho GTPase signaling, led to suppression of the TTRV30M-induced phenotype (Fig. 3B). In this study and through the identification of modifiers of TTRV30M-induced rough eye phenotype, we uncovered Rho1 and Rok as enhancers, and Rac1/2, Cdc42, Pak, LIMK, Twinstar and Chickadee as suppressors, highlighting a novel genetic interaction between TTRV30M and Rho GTPase signaling pathways with impact on actin dynamics regulation.

TTRV30M leads to axonal cytoskeleton alterations via Rho GTPases. We observed that TTRV30M disturbs axonal projection of photoreceptors (Fig. 2B). We next asked if the modifiers of TTRV30M-associated phenotype played a role in this axonal phenotype. Accordingly, we selected a suppressor (*rac1; rac2* double mutation) and an enhancer (*rho1*) and evaluated the impact of haploinsufficiency of these genes in the axonal projections of TTRV30M expressing larvae. We observed that removal of one copy of both *rac1* and *rac2* (*rac1*^{J11}*rac2*$^{\Delta}$) reverted the axonal projection phenotype (Fig. 4A,C), with only 12.5% of the larvae showing abnormal axonal projections (Fig. 4C), which is in agreement with the results observed with adult retinas. In contrast, haploinsufficiency for *rho1* led to an increase, to 88.9%, in the percentage of larvae with abnormal axonal projections, in agreement with *rho1* downregulation acting as an enhancer of TTR-mediated phenotypes (Fig. 4B,C). Of note, in this genetic background the growth cones appear even more disrupted, exhibiting a blunt-ended morphology (Fig. 4B′). Axonal projections from heterozygous larvae for *rac1*^{J11}*rac2*$^{\Delta}$ and *rho1*^{72F}

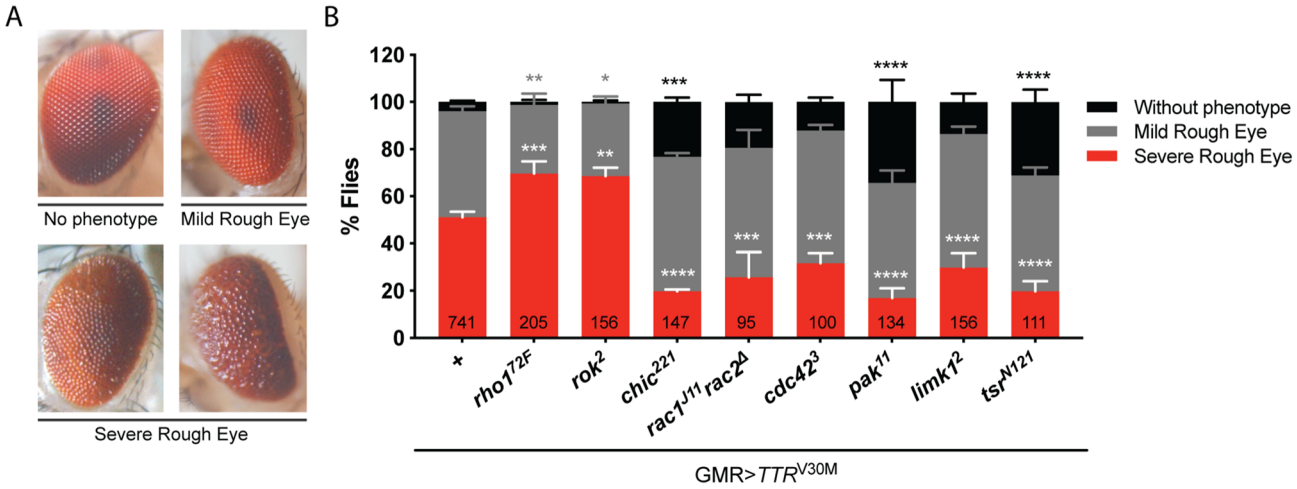

Figure 3. Candidate genetic screen with members of Rho GTPase signaling pathways. (**A**) Representative pictures of the different phenotypes observed in adult retinas and the corresponding categories: no phenotype, mild rough eye (flies with a minor or marked disorganization of the compound eye), severe rough eye (flies with highly disorganized structure of the compound eye and with bigger ommatidia). (**B**) Percentage of flies in each eye phenotype category. Flies expressing TTRV30M and lacking one copy of the gene indicated were analyzed for suppression or enhancement of the TTRV30M-associated rough eye phenotype (first column, GMR>*TTR* ^{V30M} crossed with wild-type flies). At least 3 independent experiments (total number of flies indicated in the respective column) were performed and only females were evaluated for each genotype. The results were plotted as mean ± SEM. Statistical significance determined by Two-Way ANOVA with Dunnett's multiple comparison test *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

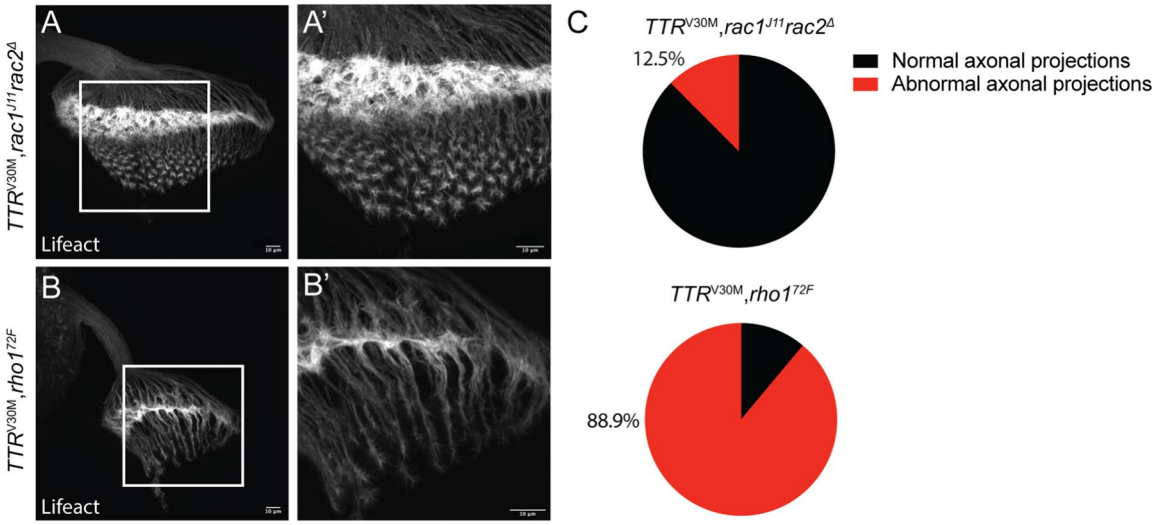

Figure 4. Rac1/2 and Rho1 are modifiers of TTRV30M-induced axonal defects. (**A**, **B**) Analysis of axonal projections in 3rd instar larvae brains expressing the actin reporter, UAS-Lifeact-ruby, under the GMR promoter. Scale bar: 10 µm. (**A**, **A′**) Haploinsufficiency for *rac1, rac2* in TTRV30M expressing larvae (*TTR* ^{V30M}*,rac1*^{J11}*rac2*$^{\Delta}$) rescues the axonal defects promoted by TTRV30M. (**B**, **B′**) Haploinsufficiency for *rho1* in TTRV30M expressing larvae (*TTR*^{V30M}*, rho1*^{72F}), enhances the axonal defects induced by TTRV30M. (**C**) Pie chart representation of the percentage of eye imaginal discs that display axonal abnormalities, as determined by qualitative analysis, in the following genotypes GMR>*Lifeact;TTR*^{V30M}*/rac1*^{J11}*rac2*$^{\Delta}$ (*TTR*^{V30M}*,rac1*^{J11}*rac2*$^{\Delta}$, n = 8); GMR>*Lifeact/rho1*^{72F}*;TTR*^{V30M} (*TTR*^{V30M}*, rho1*^{72F}, n = 9).

are indistinguishable from control larvae (GMR>*Lifeact*) (Supplementary Fig. S3A–C). These results show that TTRV30M interacts genetically with Rac1/2 and Rho1 to modify axonal behavior and actin organization.

Discussion

In this work, taking advantage of a *Drosophila* FAP model[14] we unraveled novel genetic interactions between TTRV30M and members of the Rho GTPase signaling pathways, associated with neurotoxicity. Additionally, our work describes the first genetic screen towards the identification of modifiers of TTRV30M-mediated

neurotoxicity, adding a valuable model for the identification of novel players mediating neurodegeneration in FAP.

We used a fly model based on the expression of TTRV30M, the most common TTR mutation associated with FAP[14], in photoreceptors. This model recapitulated the previously described rough eye phenotype, a critical tool to use as readout in this study. We observed that flies expressing wild-type TTR have a normal adult retina, although protein levels were similar to the ones in TTRV30M expressing flies, confirming the specific effect of the amyloidogenic mutant. In addition to the eye phenotype, we analysed lifespan and climbing activity of TTRV30M expressing flies. We observed that flies expressing TTRV30M displayed reduced lifespan and impaired climbing activity that was aggravated with aging, reflecting the progressive deleterious effect of TTRV30M. These degenerative phenotypes reflect TTR neurotoxicity and can be explained by the finding that TTRV30M is secreted from retina expressing cells. Similar results were observed with other amyloidogenic mutants, TTRL55P and TTRV14N/V16E when expressed under the same promoter[17]. According to our study, TTRV30M insoluble aggregates are detected in 1-day old flies. Since toxicity correlates mainly with soluble aggregation states, we propose that soluble TTR expressed in the retina is secreted to the hemolymph where it forms soluble aggregates inducing neurotoxicity in adjacent tissues like the brain, leading to impaired neuronal function. These results are in line with what happens in vertebrates where TTR is synthesized in liver and choroid plexus and secreted to plasma and CSF, respectively, being able to reach other tissues, as is the case of the sciatic nerve[8]. Our characterisation validated TTRV30M expressing flies as a suitable model to study TTRV30M-induced neurotoxicity, and adequate to use in genetic screens.

Our analysis showed that the TTRV30M-associated phenotype arises during larval development, as indicated by the defective axonal projections observed in TTRV30M-expressing larvae. Although this model does not exactly recapitulate FAP, as patients are asymptomatic during the development and maturation of the nervous system, the occurrence of developmental associated defects underlying disease has not been addressed to date.

Considering the impact of cytoskeleton dysfunction on neurodegenerative disorders[10], and the fact that neurotoxicity in FAP is mediated by interaction of TTR aggregates with the RAGE receptor[12], which was previously shown to be involved in the control of actin cytoskeleton remodelling[13], we performed a small-scale guided genetic screen focused on Rho GTPase signaling pathways to determine if they could act as modifiers of TTRV30M-induced neurotoxicity. Our analysis shows that downregulation of the Rho1-Rok pathway enhances TTRV30M-induced rough eye phenotype, and axonal projection defects while the downregulation of the Rac/Cdc42/Pak/LIMK pathway has the opposite effect, and acts as a suppressor of both phenotypes. RhoA/ROCK signaling has been implicated in several neurodegenerative disorders, and inactivation of the pathway, mainly through the use of ROCK inhibitors, promoted a beneficial impact on those disorders[10]. Contrarily, our results point to a negative effect of Rho1 and Rok downregulation on TTRV30M-induced neurotoxicity. Downregulation of RhoA/ROCK leads to a decrease in phosphorylation of profilin impacting on the activation of the protein. This suggests that in our settings where downregulation of Rho1/Rok aggravated TTRV30M-induced phenotype increased levels of active profilin should induce a similar effect. Accordingly, we observed a suppression of the TTRV30M-induced rough eye phenotype with the downregulation of chickadee, the *Drosophila* homolog of profilin. Importantly, profilin mutations were related with neurodegeneration in Amyotrophic lateral sclerosis (ALS)[18].

The impact of Rac on neurodegeneration is still unclear. It was shown that both upregulation and downregulation of Rac1/2 led to amelioration on different neurodegenerative disorders[19], highlighting a disease and context specific impact. Studies in *Drosophila* neurons demonstrated that activation of the Trio GEF-Rac/Cdc42/Pak/LIMK pathway lead to inhibition of axonal growth, through LIMK-mediated cofilin phosphorylation and inactivation and consequently dysregulation of actin dynamics[20]. Our data shows that downregulation of Rac1/2, Cdc42, Pak and LIMK suppress TTRV30M-induced defects, in accordance with the referred study[20]. Moreover, decreased levels of twinstar, the *Drosophila* homolog of cofilin, a LIMK substrate, also suppressed the TTRV30M-induced rough eye phenotype.

In agreement with an interaction between TTRV30M and members of the Rho GTPase family, the disorganization of the actin cytoskeleton in photoreceptor axons of TTRV30M-expressing larvae was modified by Rho1 and Rac. We observed an antagonistic effect of Rac/Cdc42 and Rho1 on the modification of TTRV30M-induced phenotype. This opposite effect might be a result from the inter-regulation between the members of the Rho GTPases family. In fact, it has been shown that RhoA and Rac can inhibit each other, mainly by acting on the regulation of GEFs and GAPs (GTPase-activating proteins)[21].

In conclusion, our data uncovered Rho GTPase signaling pathways as novel players in the molecular mechanisms through which TTRV30M potentially induces neurotoxicity. Most likely this is mediated through signaling, as we failed to detect a direct interaction between TTRV30M and Rho1, and Rac1 (Supplementary Fig. S4). Additionally, our observations suggest that Rho GTPase pathway modulators might have a beneficial impact on FAP. In the case of Alzheimer's Disease (AD), the use of ROCK inhibitors to modulate RhoA was shown to impact on disease phenotype. Moreover, NSC23766 and some nonsteroidal anti-inflammatory drugs (NSAIDs) were suggested as beneficial therapeutic options targeting Rac1 and Cdc42[22]. Concerning our data, Rac1 and Cdc42 inhibitors could be a suitable option for TTR-induced neurodegeneration. Nevertheless, future research will address the significance of Rho GTPase signaling pathways in FAP mouse models, that will contribute to increase the knowledge on the pathogenesis of the disease.

Materials and methods

Fly strains and genetics. The fly strains used in this study are described in Supplementary Table S1. Fly stocks and crosses were maintained in standard growth media, at 25 °C, unless stated otherwise. The Gal4/UAS system was used to drive expression of human TTR transgenes in the fly retina, under the control of GMR-

Gal4[23]. Transgenic flies enabling the expression of human wild type (WT) TTR (UAS-*TTR*) and a mutant and strong amyloidogenic form TTRV30M (UAS-TTR^{V30M}-HA) were previously reported[14,17]. Standard genetic techniques were used to generate the different genetic backgrounds in this study (Supplementary Table S1).

For the genetic interaction screen, GMR>TTR^{V30M} females were crossed with males from the mutant allele under test. To test interactions with genes located on the X chromosome males GMR>TTR^{V30M} were used in the cross. For control GMR>TTR^{V30M} homozygous flies were crossed with WT flies, so that there is only one copy of the UAS-TTR^{V30M} transgene in the background, similar to what is obtained after crossing GMR>TTR^{V30M} with the different mutant alleles. Crosses were performed at 25 °C and eggs were transferred and let to develop at 29 °C to enable maximum expression levels of UAS-transgenes. The retina phenotype of 1-day old flies (F1 progeny) was analyzed and flies were scored into three categories: no phenotype, mild rough eye and severe rough eye (Fig. 2A). Results were plotted as the percentage of flies in each category. Data analysis was performed with Two-Way ANOVA with Dunnett's multiple comparisons test. Adult retina phenotype was imaged in a stereomicroscope Stemi 2000 Zeiss equipped with a Nikon Digital SMZ 1500 camera, with a 50× magnification.

Lifespan assay. Recently hatched flies were allowed to mate for 2 days and separated by gender into groups of 20 flies, in a total of 100 flies per genotype. Flies were maintained at 29 °C throughout the assay. Flies were transferred into fresh vials every 2–3 days and the dead flies were scored. The survival curves were plotted as the percentage of live flies at each time point.

Climbing assay. Recently hatched flies were allowed to mate for 2 days and separated by gender into groups of 20 flies, in a total of 60 flies per genotype. Flies were maintained at 29 °C throughout the assay. To perform the climbing assay, flies were gently shaken to the bottom of the tube and allowed to climb for 20 s. The number of flies that were able to climb up to 5 cm was counted and expressed as a percentage, which refers to climbing ability. Each set of flies was tested three times at each time point (1, 5, 10, 15, and 20 days after hatching). Results are plotted as mean ± SEM. Data analysis was performed with Two-Way ANOVA with Tukey's multiple comparisons test.

Extract preparation for protein analysis. For analysis of TTR expression levels six heads from1-day old flies, per genotype, were homogenized in 20 µL of Laemmli Sample Buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 100 mM β-mercaptoethanol, 10% glycerol and 0.1% bromophenol blue) and snap frozen. Prior to SDS-PAGE analysis samples were heated at 100 °C for 5 min and centrifuged at 13,000 rpm for 5 min.

For analysis of soluble and insoluble fractions thirty adult fly heads, and fifty third instar larval brain and imaginal discs, were used per genotype. Tissue homogenization was performed in 100 µL of extraction buffer (PBS with 1% triton X-100 (Sigma-Aldrich), protease inhibitors (Protease Inhibitor Mix GE Healthcare, 80-6501-23, 1:100) and sodium orthovanadate 1 mM) for 30 s. Extracts were centrifuged at 15,000*g* for 10 min at 4 °C and supernatant was collected as the Triton-soluble fraction. The pellet was washed 3 times with extraction buffer and resuspended in 100 µL of 50 mM Tris pH 7.5 with 4% SDS and protease inhibitors followed by sonication. Extracts were centrifuged at 15,000*g* for 10 min at 4 °C and supernatant was collected as the Triton-insoluble fraction. SDS-PAGE and western blot analysis were performed as described below.

Immunoprecipitation. Two hundred flies per genotype were collected, frozen with liquid nitrogen and vortexed for 15 s to separate fly heads. Fly heads were homogenized in PBS with 0.3% triton X-100 (Sigma-Aldrich), protease inhibitors (EDTA-free Protease Inhibitor Cocktail Sigma-Aldrich 1:100) and sodium orthovanadate 1 mM). Head lysates were centrifuged at 10,000 rpm for 20 min at 4 °C, and the supernatant was pre-cleared prior to use in immunoprecipitation. To pre-clear lysates, 250 µg of head lysate were incubated with 25 µL of magnetic beads (Dynabeads™ Protein G, Novex) overnight. Pre-cleared head lysate was incubated with bead-antibody (Ab) complex overnight at 4 °C. 2 µg of anti-human TTR (A0002, DAKO) coupled to 25 µL of magnetic beads were used. Magnetic beads—pelleted TTR—protein complexes were eluted with 2× Laemmli Sample Buffer. SDS-PAGE and western blot analysis were performed as described below.

Western blot. Protein extracts were applied on 15% SDS-PAGE gels and transferred to a 0.45 µm nitrocellulose membrane (Amersham). Membrane was blocked in 5% non-fat dry milk in TBS-T [TBS with 0.05% Tween (Sigma-Aldrich)] for 1 h, prior to overnight incubation at 4 °C with primary antibodies: rabbit anti-hTTR (A0002, DAKO, 1:1,000), mouse anti-α-Tubulin (T5168, clone B512, Sigma-Aldrich, 1:100,000), mouse anti-GAPDH (G-9, sc365062, Santacruz, 1:500), mouse anti-Rho1 (P1D9, Developmental Studies Hybridoma Bank, 1:250), mouse anti-Rac1 (23A8, ab33186 Abcam, 1:2000) in blocking buffer. Incubation with horseradish peroxidase-labeled secondary antibodies was performed for 1 h at room temperature in blocking buffer. Blots were developed using the enhanced chemiluminescence western blot substrate (Bio-Rad).

Immunohistochemistry. Third instar larvae were collected and dissected in ice cold PBS. Larval eye-antenna imaginal discs and brain were dissected, fixed in 3.7% formaldehyde in PBS for 20 min and mounted in 50% glycerol in PBS. Larval samples fixed and processed for immunohistochemistry were washed three times in PBT 0.3% [1× PBS + 0.3% Triton X-100 (Sigma-Aldrich)] and incubated with primary antibodies: rabbit anti-HA (Abcam Ab9110, 1:50), rat anti-DCAD2 (Developmental Studies Hybridoma Bank, 1:100), mouse anti-PDF (C7, Developmental Studies Hybridoma Bank, 1:100) in PBT 0.3%, overnight at 4 °C. Secondary antibodies (Alexa anti-rabbit 568 (A10042), Alexa anti-rat 647 (A21247), Alexa anti-mouse 488 (A21202) ThermoFisher Scientific) were diluted in PBT 0.1% (1× PBS + 0.1% Triton X-100) and incubated for 3 h at room temperature.

Discs were mounted in 50% glycerol in PBS. For F-actin labeling, Lifeact expressing larvae were imaged using an inverted laser scanning confocal microscope (Leica TCS SP5II) with a 63× objective. Image stacks with 0.3–0.5 µm of z-step size were acquired of each imaginal disc and brain and z-stacks of interest were projected using Fiji software. The percentage of eye-imaginal discs with abnormal axonal projections was determined by qualitative analysis of the images. At least 7 imaginal discs were analyzed per genotype.

Brains from adult flies (1 day old) were dissected in cold PBS and fixed in 3.7% formaldehyde in PBS for 30 min. Samples were washed three times in PBT 0.5% [1 × PBS + 0.5% Triton X-100 (Sigma-Aldrich)] and incubated in blocking buffer (PBT 0.5% + 0.5% BSA + 0.5% FBS) for 1 h 30 min at room temperature. Incubation with primary antibodies: mouse anti-PDF (C7, Developmental Studies Hybridoma Bank, 1:100) was performed in blocking buffer overnight at 4 °C. Secondary antibody (Alexa anti-mouse 488 (A21202) ThermoFisher Scientific) was diluted in PBT 0.5% and incubated for 3 h at room temperature. Tissues were mounted in Fluoromount-G™ Mounting Medium (ThermoFisher Scientific). Whole-mount adult brain samples were imaged using an inverted laser scanning confocal microscope (Leica TCS SP5II) with a 40× or 63× objectives. Image stacks with 0.7–1 µm of z-step size were acquired of each sample and the z-stacks of interest were projected using Fiji software.

Whole-mount adult brains immunostained for PDF-positive neurons were imaged as previously described and z-stacks projected into a single image. Quantification was performed in grayscale images and the areas of the axonal arborization and of the brain hemisphere were measured. For that, the polygon selection tool of the Fiji software was used and the limits of the axonal projection and the brain hemisphere were drawn and the area calculated. The normalized area represents the area of the PDF axonal projections divided by the area of the respective brain hemisphere. 7 brains from control (GMR-G4) flies and 11 brains from GMR>*TTR*^{V30M} flies were analysed.

Scanning electron microscopy (SEM). For SEM analysis, 1-day old flies were dehydrated through incubation in ethanol series (25%, 50%, 75%, 100%), incubated with hexamethyldisilazane (HMDS, Sigma), air-dried, and mounted in SEM stubs and coated with Au/Pd thin film, by sputtering using the SPI Module Sputter Coater equipment. Imaging was performed using a High resolution (Schottky) Environmental Scanning Electron Microscope with X-ray microanalysis and Electron Backscattered Diffraction analysis: Quanta 400 FEG ESEM/EDAX Genesis X4M. The images were acquired with 400× and 1,000× magnification.

Statistical analysis. The statistical analysis was performed with GraphPad Prism software version 8. The assays with more than one experiment are represented as mean ± SEM (standard error of the mean). Data comparison between groups was performed using Two-Way ANOVA with Dunnett's multiple comparisons test, with Tukey's multiple comparisons test or Sidak's multiple comparison test, as referred in each experiment. $p < 0.05$ was considered statistically significant.

Data availability

Raw data generated during this study is available upon request.

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Author contributions

C.S.L. and M.A.L. designed the study. M.I.O.S. performed experiments and data analysis. M.I.O.S., C.S.L. and M.A.L. wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Transthyretin Interacts with Actin Regulators in a *Drosophila* model of Familial Amyloid Polyneuropathy

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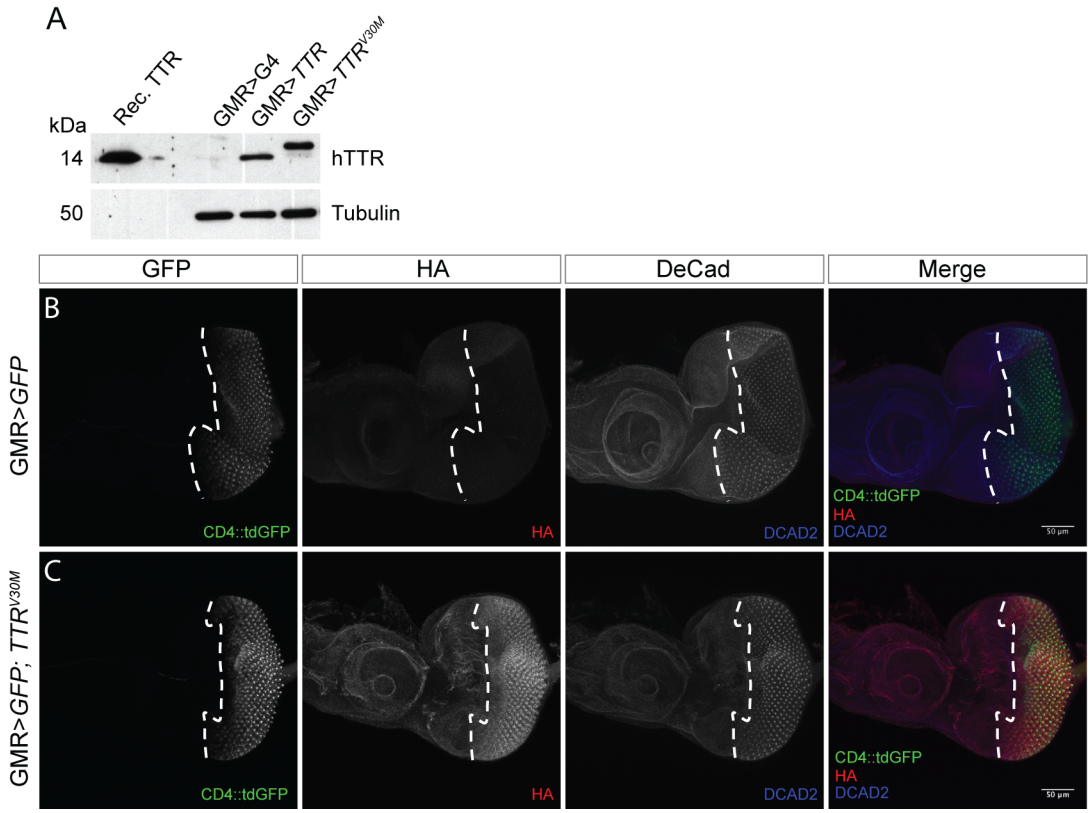
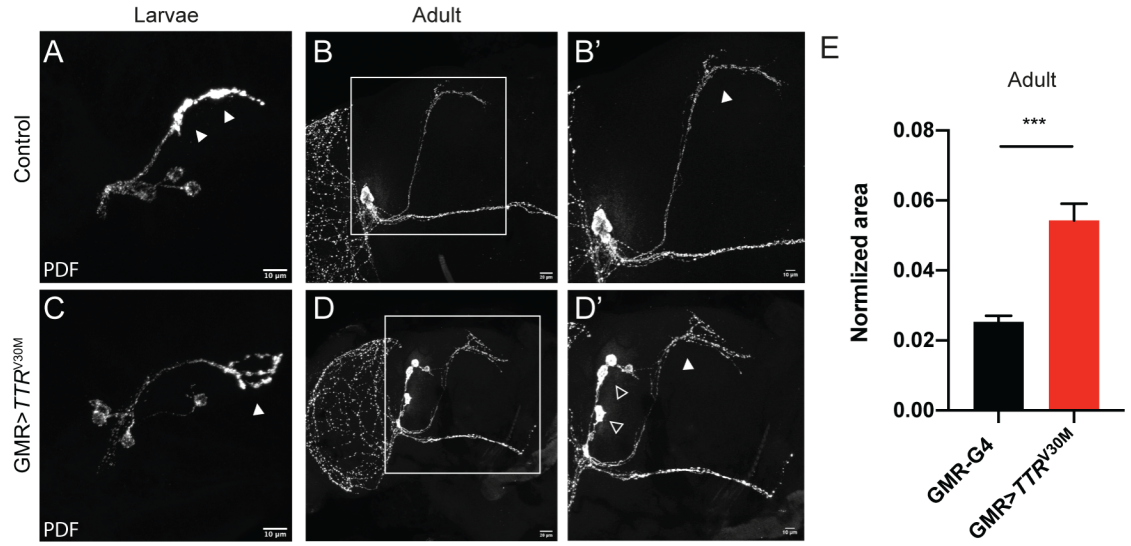
Supplementary Information

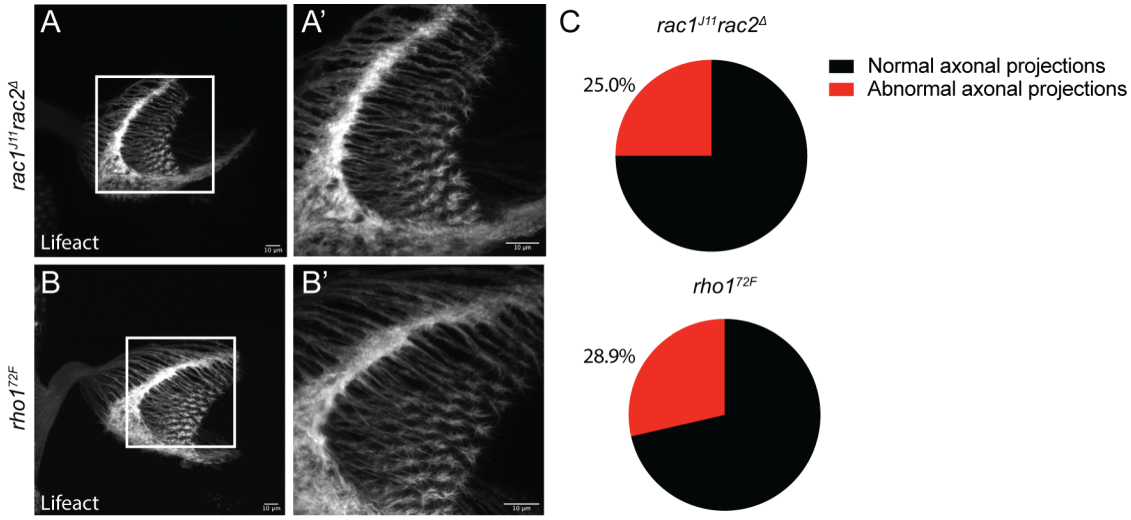
Supplementary Figures



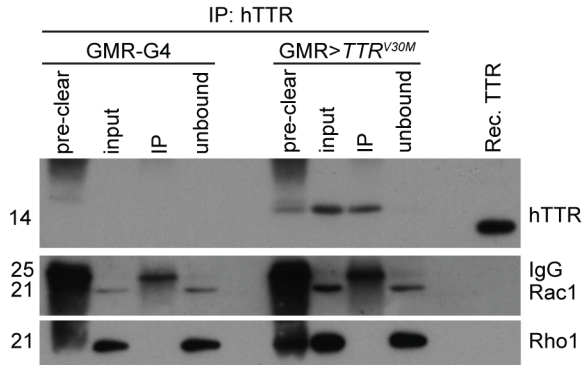
Supplementary Figure S1. Expression of TTR in the fly developing eye and retina. (A) Western blot of 6 fly head extracts from 1-day-old flies of the following genotypes: GMR>*G4*, GMR>*TTR*, GMR>*TTR*^{V30M}. The presence of a HA tag on TTRV30M results in a band with higher molecular weight. Recombinant TTR (Rec. TTR) was used as positive control. Tubulin was used as loading control. **(B, C)** Immunohistochemistry on 3rd instar eye-imaginal discs of the following genotypes: GMR>*GFP* (B), GMR>*GFP*;*TTR*^{V30M} (C). Expression of CD4::tdGFP (green) was used to define the domain of expression of GMR-Gal4 promoter. HA (red) was used to detect TTRV30M. DECAD2 staining (blue) was used to visualize epithelia morphology. Scale bar: 50µm.



Supplementary Figure S2. TTRV30M expression in photoreceptors affects axonal arborization of PDF neurons (A, C) Immunohistochemistry of 3rd instar larval brains of control (GMR-G4) and GMR-*TTR*^{V30M}. Scale bar: 10µm **(B)** Immunohistochemistry on brains from 1-day old control flies (GMR-G4). Scale bar: 20µm **(B')** High magnification of (B) showing the normal arrangement of axonal arborization (white arrowheads). Scale bar: 10µm **(D)** Immunohistochemistry of PDF expressing neurons in adult brains from 1-day old GMR>*TTR*^{V30M} flies. Scale bar: 20µm **(D')** High magnification of (D) showing increased axonal arborization (white arrowheads) and the disorganized spatial arrangement of cell bodies (open arrowheads). Scale bar: 10µm **(E)** Quantification of the area occupied by the axonal arborization of the PDF-positive neurons normalized by the respective area of the brain hemisphere, in 1-day old flies. Statistical significance determined by Two-Way ANOVA with Sidak's multiple comparisons test.

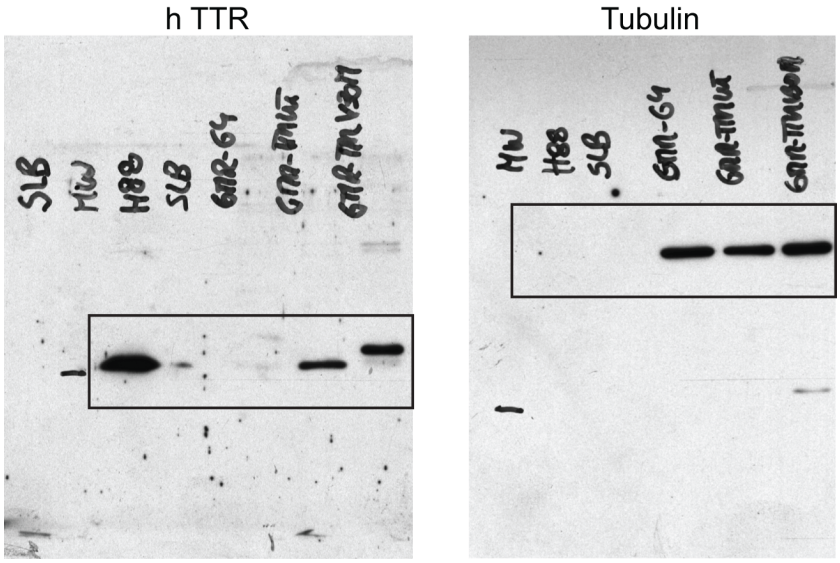


Supplementary Figure S3. Haploinsufficiency for *rho1* or *rac1*^{J11}*rac2*$^{\Delta}$ does not affect axonal projections organization. (A, B) Analysis of axonal projections in 3rd instar larvae brains expressing the actin reporter, UAS-Lifeact-ruby, under the GMR promoter. Scale bar: 10µm. **(A, A')** GMR>*Lifeact;rac1*^{J11}*rac2*$^{\Delta}$ (*rac1, rac2*) larvae do not display defects in axonal projections. **(B, B')** Axonal defects were not detected in GMR>*Lifeact/rho1*^{72F} (*rho1*^{72F}) larvae. **(C)** Pie chart representation of the percentage of larvae with abnormal axonal projections evaluated qualitatively. GMR>*Lifeact;rac1*^{J11}*rac2*$^{\Delta}$ (*rac1*^{J11}*rac2*$^{\Delta}$, n=8); GMR>*Lifeact/rho1*^{72F} (*rho1*^{72F}, n=7).

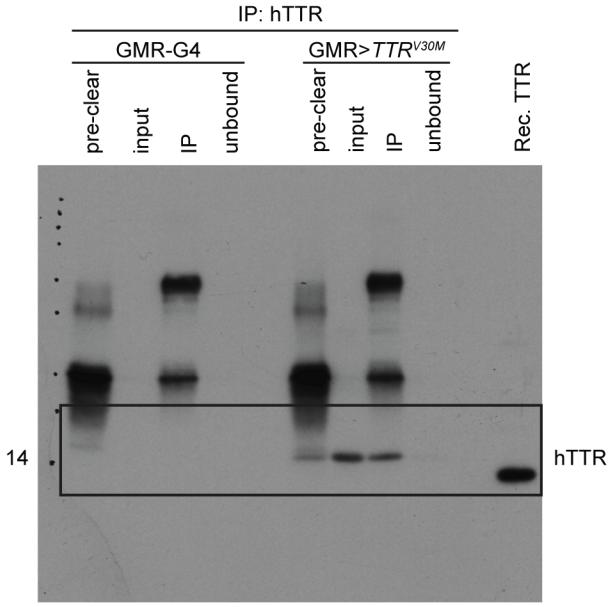


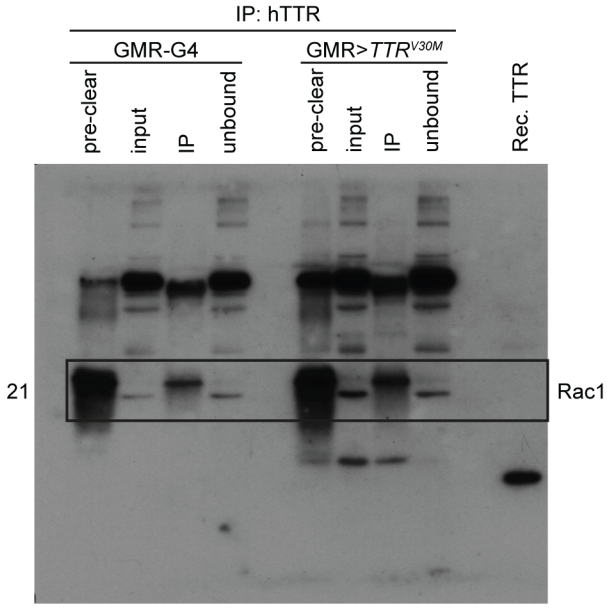
Supplementary Figure S4. Rac1 and Rho1 do not co-immunoprecipitate with TTRV30M. Co-immunoprecipitation of Rac1and Rho1 using the anti-human TTR antibody, in extracts from control (GMR-G4) and GMR>*TTR*^{V30M} adult fly heads. Lanes: Pre-clear, pellet from extract beads incubation showing unspecific binding to the magnetic beads. Input, pre-cleared extract used for the immunoprecipitation assay. IP, proteins precipitated with anti-TTR-beads complex. Unbound, extract not bound to anti-TTR-beads complex. TTR is present in the input and recovered in the IP fraction. Rac1 and Rho1 are present in the input but are not recovered in the IP fraction.

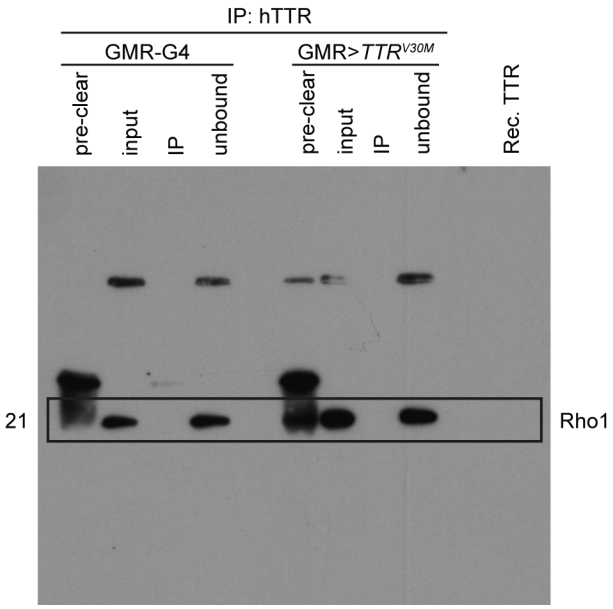
Full-length blots Figure S1A



Full-length blots Figure S4







Supplementary Table

Table S1. List of fly strains used and generated in this study with respective genotype and source.

Stock	Genotype	Source
Wild-type	w^{1118}	in-house
GMR-*GAL4*	GMR-Gal4/GMR-Gal4	(Hay *et al.*, 1997)
Gal80^{ts}	P{tubP-GAL80[ts]}2	Bloomington 7017
UAS-*Lifeact*	y^1 w*; P{UAS-Lifeact-Ruby}VIE-19A	Bloomington 35545
UAS-*Lifeact*	w*; sna^{Sco}/CyO; P{UASt-Lifeact-RFP}3	Bloomington 58362
UAS-*mCD4-GFP*	w^{1118};PBac{UAS-CD4-tdGFP}VK00033	Bloomington 35836
UAS-*TTR*	w^{1118}; P{UAS-TTR^{wt}}	(Pokrzywa *et al.*, 2007)
UAS-*TTR^{V30M}*	w^{1118}; P{UAS-$TTR^{V30M-HA}$}	(Berg *et al.*, 2009)
Balancers	w^{1118};Sp/CyO;MKRS/TM6b, Tb	in-house
GMR>*TTR*	GMR-Gal4/GMR-Gal4;+/+;UAS-TTR/UAS-TTR	This study (Fig. 1, S1)
GMR>*TTR^{V30M}*	GMR-Gal4/GMR-Gal4;+/+;UAS-TTR^{V30M}/UAS-TTR^{V30M}	This study (Fig. 1-4, S1, S2)
GMR>*GFP*	GMR-Gal4/GMR-Gal4;+/+;UAS-GFP/UAS-GFP	This study (Fig. 1, S1)
GMR>GFP;*TTR^{V30M}*	GMR-Gal4/+;UAS-GFP/+; UAS-TTR^{V30M}/+	This study (Fig. S1)
GMR>*Lifeact;TTR^{V30M}*	GMR-Gal4/+;UAS-Lifeact/+;UAS-TTR^{V30M}/+	This study (Fig. 2, 4, S2)
Cdc42^3	w^1 sn^3 $Cdc42^3$/FM6	Bloomington 7337
Pak^{11}	Pak^{11}/TM3, Sb^1	Bloomington 8810
Rac1$^{\Delta}$ Rac2$^{\Delta}$	y^1 w*; $Rac1^{J11}$ $Rac2^{\Delta}$ P{FRT(whs)}2A/TM6B, Tb+	Bloomington 6677
Rho1^{72F}	y^1 w*; $Rho1^{72F}$/CyO	Bloomington 7326
Rok^2	y^1 w^{1118} Rok^2 P{neoFRT}19A/FM7c	Bloomington 6666
Limk1^2	w* $LIMK1^2$	Bloomington 59033
tsr^{N121}	w*; P{FRT(w^{hs})}G13 tsr^{N121}/CyO	Bloomington 9109
chic^{221}	$chic^{221}$ cn^1/CyO; ry^{506}	Bloomington 4892

CHAPTER I

Cofilin pathology is a new player on α-synuclein-induced spine impairment in models of hippocampal synucleinopathy

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Abstract

Cognitive dysfunction and dementia are presently recognized as major complications in synucleinopathies, namely in Dementia with Lewy Bodies (DLB) and Parkinson's disease with dementia (PDD). In these disorders, α-Synuclein (αSyn) accumulation induces synaptic dysfunction in brain regions which coordinate cognition, namely the hippocampus. However, the molecular mechanisms underlying αSyn impact on the hippocampal neuronal function remain elusive. To address this question, we analyzed the effect of overexpression of αSyn on hippocampal neurons. We observed that αSyn induces the dysregulation of the actin-binding protein cofilin and its assembly into rod structures in a mechanism mediated by the cellular prion protein (PrP^C) and the chemokine receptor CCR5. Moreover, we unraveled cofilin pathology as mediator of αSyn-induced dendritic spine impairment in hippocampal neurons. Importantly, in a synucleinopathy mouse model with cognitive impairment we validated cofilin dysregulation and synaptic impairment at the same age when cognitive deficits were observed. Our data supports cofilin as a novel player on hippocampal synaptic dysfunction triggered by αSyn on Lewy Body dementias.

Introduction

α-Synuclein (αSyn) is well-known due to its involvement in Parkinson's disease (PD), a neurodegenerative disorder mainly characterized by motor dysfunction (Jankovic, 2008), in which the major hallmarks are the formation of neuronal αSyn-containing inclusions, known as Lewy Bodies (LBs), and the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Braak et al., 2003a; McCann et al., 2014). Nevertheless, αSyn is implicated in other synucleinopathies namely Lewy Body dementias (LB dementias), which include Dementia with Lewy Bodies (DLB) and Parkinson's disease with dementia (PDD). These two disorders present similar symptomatology, being mainly distinguished by the time at which dementia occurs. In DLB, cognitive impairment precedes parkinsonism or begins within a year of parkinsonism, whereas in PDD, parkinsonism generally precedes cognitive impairment by more than 1 year (Yang and Yu, 2017). While DLB accounts for ~30% of all age-related dementias, in PD, 20–40% of the patients have cognitive impairments at disease onset, and ~80% of the patients develop PDD with the course of the disease (Hely et al., 2008). Despite the impact on patients, no treatments have been proven to slow or stop disease progression in LB dementias, and the pathophysiologic mechanisms underlying these disorders require further investigation.

LB dementias have been linked to increased expression/aggregation of αSyn in the hippocampal brain region leading to neuronal dysfunction. Locus multiplications of the *SNCA* gene have been described in LB dementia cases, whose severity of cognitive impairment and age of onset correlates with the copy number of the *SNCA* gene (Ikeuchi et al., 2008). The hippocampus, a major brain component with key roles in memory and learning, is one of the most vulnerable regions affected by αSyn pathology (Yang and Yu, 2017). In this respect, hippocampal volume loss was observed in DLB and PDD patients, but not in cases of PD with normal cognition (Camicioli et al., 2003). Moreover, increased levels of LB pathology were observed in the hippocampus of LB dementias (Walker et al., 2019). These observations suggest that the impact of αSyn on the hippocampus may underlie cognitive deficits and dementia observed in LB dementias. Additionally, the reported synaptic dysfunction in the hippocampus of mouse models of synucleinopathies evidence the link between cognitive deficits and hippocampal LB pathology.

In this study, we aimed at characterizing mechanisms downstream of αSyn hippocampal pathology underlying synaptic impairment and cognitive dysfunction in LB dementias. We focused on the actin cytoskeleton, not only due to its critical role in synaptic function, but also because a link between this cell component and αSyn has been increasingly supported by the literature (Bras et al., 2018; Oliveira da Silva and Liz, 2020).

One of the consequences of the dysregulation of the actin cytoskeleton in neurodegenerative disorders is the formation of cofilin-actin rods. These are structures composed by bundles of cofilin-saturated actin filaments, which result from the dysregulation of the actin binding protein cofilin *via* localized hyperactivation by dephosphorylation and oxidation, and that were shown to block intracellular trafficking and induce synaptic loss in hippocampal neurons (Cichon et al., 2012). Cofilin-actin rods have been mainly implicated in cognitive impairment in Alzheimer's disease (AD) (Minamide et al., 2000; Rahman et al., 2014) and synaptic dysfunction after ischemic stroke (Shu et al., 2018; Won et al., 2018). Importantly, decreasing either cofilin dephosphorylation or the total levels of cofilin expression was effective in the amelioration of cofilin pathology and cognitive deficits in the Aβ-overproducing mouse model of AD (Woo et al., 2015a; Woo et al., 2015b; Deng et al., 2016). Additionally, in a rat model of ischemic stroke, which presents rod formation in the peri-infarct area, the overexpression of active LIM kinase (LIMK), which phosphorylates cofilin promoting its inactivation, resulted in decreased rod formation and rescue of synaptic activity (Shu et al., 2018). These data suggest that modulation of cofilin pathology is beneficial to preserve synaptic function and prevent cognitive deficits in these neurological disorders.

Here we show that αSyn induces cofilin pathology in hippocampal neurons *via* a cellular prion protein (PrP^C) and chemokine receptor CCR5-dependent pathways. Moreover, cofilin dysregulation mediates dendritic spine impairment in response to αSyn. Based on the present data, we propose that αSyn-induced cofilin pathology may underlie synaptic dysfunction and cognitive impairment in LB dementias.

Results

αSyn induces cofilin-actin rod formation in hippocampal neurons

In order to characterize mechanisms underlying hippocampal dysfunction in LB dementias, we analyzed the effect of overexpressing wild-type (WT) αSyn in primary cultures of hippocampal neurons to establish a scenario of increased levels and aggregation of αSyn which were shown to induce synaptic dysfunction (Scott et al., 2010; Lazaro et al., 2017). Rat hippocampal neurons were infected at DIV4 with lentivirus encoding for WT αSyn-IRES-GFP or IRES-GFP as control. Overexpression of αSyn, which was highly phosphorylated at Ser129, was confirmed by western blot and immunostaining of DIV7 transduced neurons (Figure 1A and B). Similar analyses at DIV14, a time point when endogenous αSyn is already expressed and enriched in presynaptic terminals (Murphy et al., 2000), allowed a quantitative analysis of αSyn overexpression relatively to the levels of endogenous protein. In DIV14 transduced neurons, αSyn levels were increased approximately 3-fold when compared to control cells, with similar levels of GFP expression as determined by western blot (Figure S1A and B). Immunocytochemistry revealed the presence of endogenous αSyn, without detection of αSyn pS129, in control cells (Figure S1C). This result is in accordance with the phosphorylation levels of αSyn pS129 in healthy brains, which correspond to about 4% of the total protein (Fujiwara et al., 2002). In the case of αSyn-transduced neurons, αSyn was extensively phosphorylated at Ser129, a pathologic-associated modification of the protein, mimicking the aberrant accumulation of αSyn pS129 in the brain of patients with LB diseases (Anderson et al., 2006) (Figure S1C). These results confirm that we have successfully established a cell system of αSyn hippocampal pathology.

Cofilin-actin rods are one of the features associated with hippocampal pathology in response to Aβ, being implicated in synaptic impairment and cognitive dysfunction in AD (Woo et al., 2015a). We hypothesized whether αSyn would exert a similar effect in the context of hippocampal pathology in LB dementias. Using our established cell system of αSyn-induced hippocampal pathology, we demonstrated that DIV7 αSyn-overexpressing neurons presented a significant 2.5-fold increase in the percentage of neurons presenting rod formation when compared to control neurons (Figure 1C and D). Interestingly, the percentage of neurons with rods in αSyn-transduced neurons was approximately of 23%, a similar value to the one reported for Aβ-induced rod formation in rat hippocampal neurons (Maloney et al., 2005; Davis et al., 2011). We also quantified rod index, which reflets the total number of rod structures divided by the number of neuronal nuclei, and we found a

2.5-fold increase in rod index in neurons expressing αSyn (Figure 1C and E). Cofilin-actin rods were described to block the intracellular trafficking and to be able to reach a dimension sufficiently large to occlude the neuronal processes. In this regard, we could observe in the αSyn-expressing neurons that neurites containing cofilin-actin rods are devoid of βIII-tubulin staining, suggesting a disturbance of the cytoskeleton integrity in rod regions (Figure 1C, insets).

In order to validate rod formation in a more relevant context we transduced αSyn in hippocampal slice cultures. The use of these slices better represents a physiologic environment since we are using the whole brain region of interest, in this case the hippocampus, what allows to analyze the neuronal response to the stimuli in its cellular environment. Analysis of hippocampal slices infected with WT αSyn-IRES-GFP lentivirus revealed a tendency to increased rod formation, when compared to IRES-GFP transduced slices (Figure 1F and G).

Our results confirm that αSyn induces cofilin pathology, *via* cofilin-actin rods formation, in hippocampal neurons.

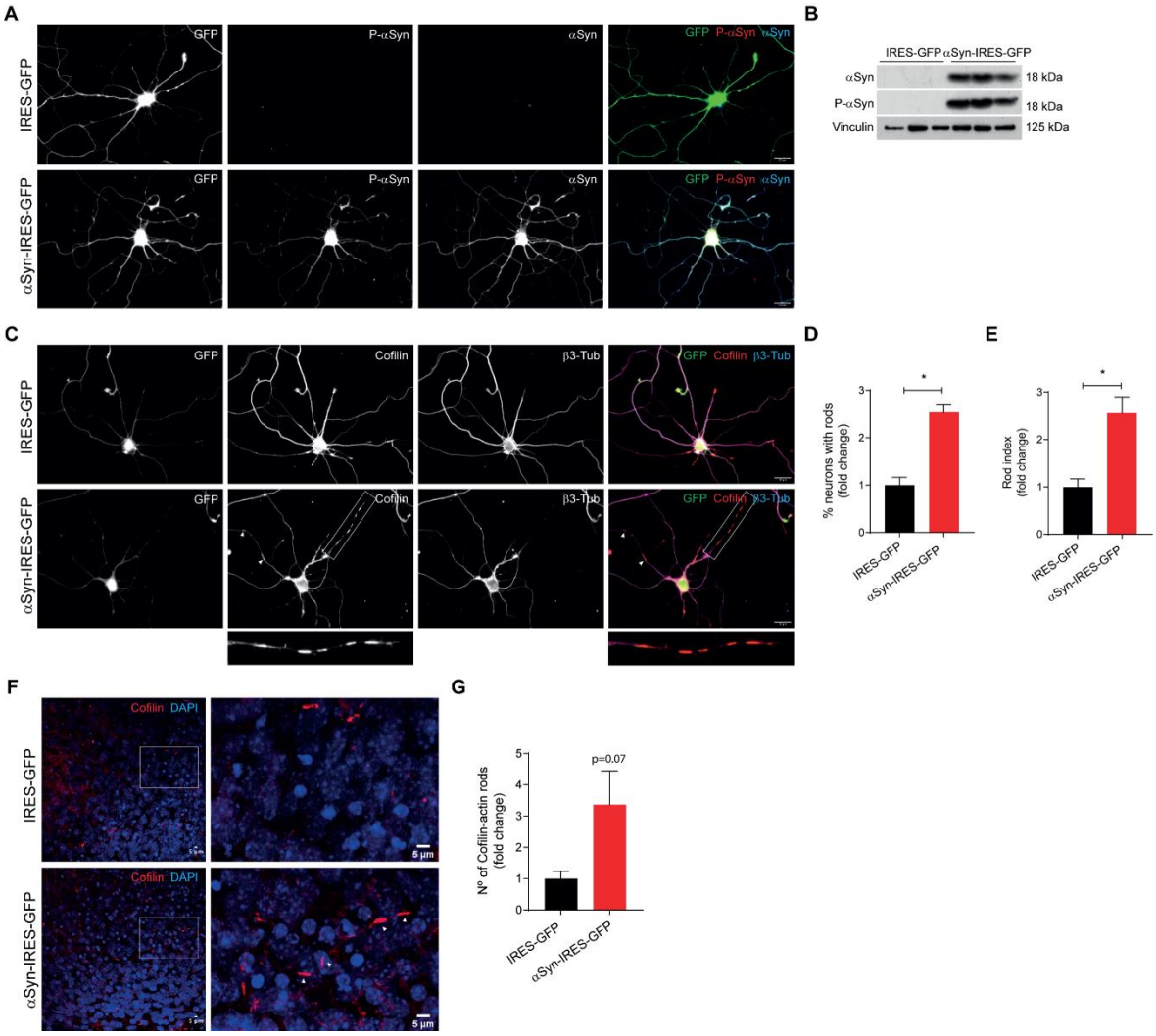


Figure 1 - αSyn overexpression induces cofilin-actin rods formation in dissociated hippocampal neurons. (A-E) Primary hippocampal neurons were infected at DIV4 with IRES-GFP or WT αSyn-IRES-GFP lentivirus and analyzed at DIV7. **(A)** Representative images of infected hippocampal neurons immunostained with αSyn pS129 (red) and αSyn (blue). Scale bar: 20 µm. **(B)** Western blot analysis of αSyn and αSyn pS129 levels in transduced hippocampal neurons. Vinculin was used as loading control. Data represent mean±SEM (n=3 replicates/treatment). **(C)** Representative images of infected hippocampal neurons immunostained with cofilin (red) and β3-tubulin (blue). Scale bar: 20 µm. Boxes indicate cofilin-actin rod structures in αSyn-treated neurons. **(D)** Quantification of the percentage of neurons with rods (shown as fold change relative to control) relative to C. Data represent mean±SEM (n=2-3 replicates/treatment with ≥100 neurons/replicate). *p<0.05 by Student's *t* test. **(E)** Quantification of rod index (shown as fold change relative to control) relative to C. Data represent mean±SEM (n=2-3 replicates/treatment with ≥100 neurons/replicate). *p<0.05 by Student's *t* test. **(F)** Representative images of hippocampal slices from WT mice treated with IRES-GFP or WT αSyn-IRES-GFP lentivirus at DIV9 and immunostained for cofilin (red) and DAPI (blue) at DIV15. Insets represent the respective zoom-ins noted by the white box and arrows denote rod structures. **(G)** Number of cofilin-actin rods relative to F. Data represent mean±SEM (n=2-3 slices/treatment).

αSyn-induced rod formation occurs via PrP^C and CCR5

Having confirmed αSyn-induced rod formation in hippocampal neurons, we aimed at dissecting the molecular mechanism involved in this process. In AD, Aβ-induced formation of rods occurs *via* a PrP^C-dependent pathway leading to NADPH oxidase (NOX) activation (Walsh et al., 2014), which culminates in the localized dysregulation of cofilin activity *via* oxidation and dephosphorylation. PrP^C is an outer leaflet membrane protein which serves as a co-receptor for several molecular partners as is the case of Aβ (Walsh et al., 2014). Being PrP^C exposed to the extracellular environment, rod formation mediated by the co-receptor requires the insult to exist in the extracellular milieu. Considering this, several reports have described the physiological presence of αSyn in the extracellular fluids such as CSF and blood plasma, as well as its presence in culture media from αSyn-overexpressing cells (Borghi et al., 2000; El-Agnaf et al., 2003). Although in our setup we overexpressed αSyn, what might suggest a solely intracellular role, we have observed the presence of the protein in the extracellular medium by dot blot analysis (Figure S2). Additional analysis with a cellulose acetate membrane, which retain protein aggregates, did not detect αSyn aggregated species (data not shown), suggesting that the culture media of αSyn transduced hippocampal neurons contains soluble forms of αSyn species, such as monomers or oligomers. Based on these observations, we analyzed whether αSyn released from transduced neurons had an impact in rod formation. Addition of αSyn-containing media for 48 h to DIV5 WT hippocampal neurons, which have no detectable levels of endogenous αSyn, induced a significant 2.6-fold increase in the percentage of neurons with rods, when compared to cells treated with control media, with approximately 20% of the neurons presenting rod formation (Figure 2A and B). Interestingly, the exogenous addition of recombinant αSyn monomeric and oligomeric species had a similar effect in rod formation (Figure 2C). Knowing that αSyn is able to induce rod formation extracellularly, we addressed whether, similarly to Aβ, αSyn could act *via* PrP^C. In order to test this hypothesis, we overexpressed αSyn using our established lentiviral strategy in hippocampal neurons from either WT or PrP^C KO mice. αSyn overexpression induced rods in neurons from WT mice, leading to a 2.3-fold increase in the percentage of neurons forming rods when compared to control transduced neurons. In the case of PrP^C KO neurons, αSyn was not able to induce rod formation (Figure 2D). Our data confirm a common molecular mechanism for rod formation between αSyn and Aβ, involving PrP^C.

A recent report have showed that chemokine receptor inhibitors significantly reduced rod formation in neurons in response to gp120, an HIV-derived envelop protein (Smith et al., 2021). Interestingly, the same study showed that chemokine receptors, namely CXCR4

and CCR5, might play a role in the Aβ dimers/trimers ($A\beta_{d/t}$)-induced rod formation pathway as the use of chemokine receptor inhibitors abolished cofilin-actin rod formation triggered by $A\beta_{d/t}$ (Smith et al., 2021). Taking into consideration that we have witnessed a similar behavior between Aβ and αSyn in hippocampal rod pathology, we addressed whether chemokine receptors could also play a role in αSyn-induced rod formation. To study this, we treated αSyn overexpressing hippocampal neurons for 24 h with 50 pM RAP-103, a CCR5 inhibitor. At DIV7, we observed an increased rod formation in WT αSyn-IRES-GFP transduced neurons, given by a 2.5-fold increase in the percentage of neurons with rods when compared with control IRES-GFP transduced neurons (Figure 2E), which was completely abolished by RAP-103 (Figure 2E). We also analyzed mature neurons overexpressing αSyn and treated with RAP-103. In these neurons we quantified rod index, as in mature neurons the percentage of neurons with rods is extremely difficult to quantify due to the complexity of the neuronal network, and observed that the use of the CCR5 inhibitor RAP-103 completely abolished rod formation induced by αSyn (Figure S3).

Overall, our findings suggest a common molecular mechanism between Aβ, gp120 and αSyn in triggering rod formation in hippocampal neurons what might underlie synaptic dysfunction, hippocampal pathology and cognitive decline observed in these diseases.

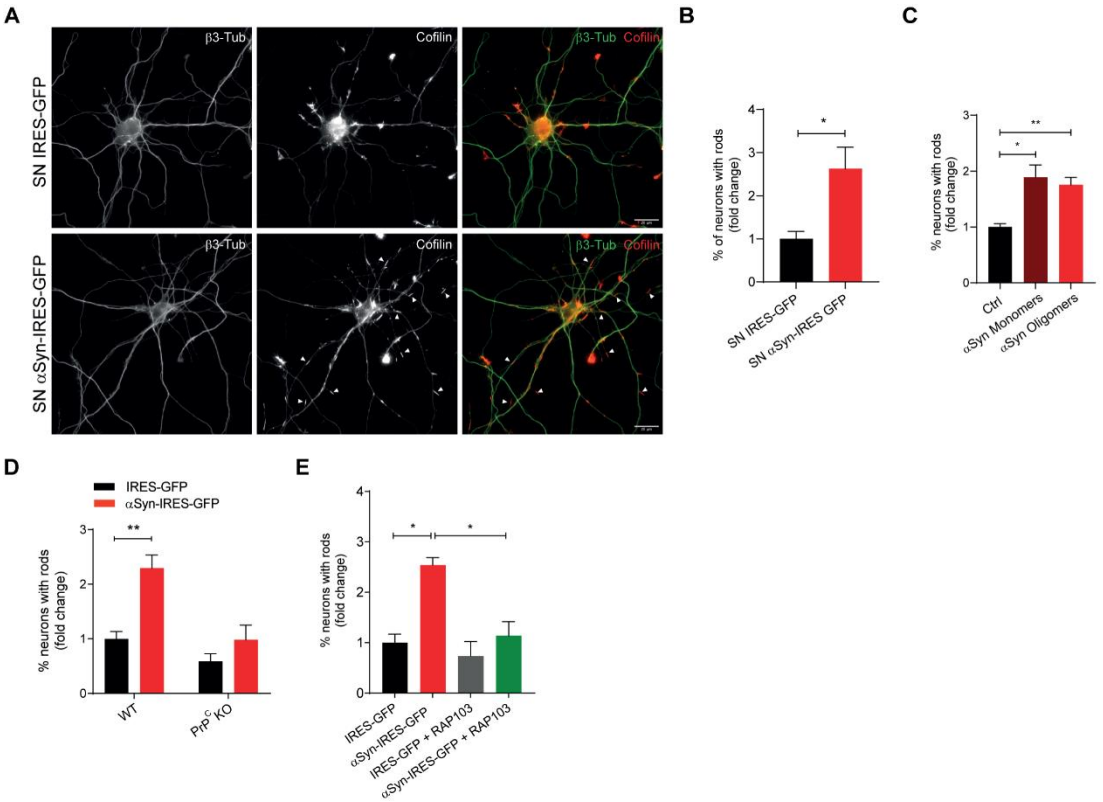


Figure 2 – PrP^C and CCR5 are involved in αSyn-induced rod formation. (A) Representative images of DIV7 hippocampal neurons previously treated for 48 h with supernatants from cultured

IRES-GFP or WT αSyn-IRES-GFP infected neurons. Immunostaining of β3-tubulin (green) and cofilin (red). Scale bar: 20 μm. **(B)** Quantification of the percentage of neurons with rods (shown as fold change relative to control) relative to A. Data represent mean±SEM (n=4 replicates/treatment with ≥100 neurons/condition). *p<0.05 by Student's *t* test. **(C)** Percentage of neurons with rods (shown as fold change relative to control) on DIV7 hippocampal neurons pre-treated with control (PBS), αSyn monomers (0.5 μM) or αSyn oligomers (0.5 μM) for 24h. Data represent mean±SEM (n=3 replicates/treatment with ≥100 neurons/condition). *p<0.05, **p<0.01 by Student's *t* test. **(D)** Hippocampal neurons from WT or PrP^C KO mice were infected at DIV4 with IRES-GFP or WT αSyn-IRES-GFP lentivirus and the percentage of neurons with rods (shown as fold change relative to control) was analyzed at DIV7. Data represent mean±SEM (n=5-6 replicates/treatment with ≥100 neurons/replicate). **p<0.01 by Student's *t* test. **(E)** Primary hippocampal neurons were infected with IRES-GFP or WT αSyn-IRES-GFP lentivirus at DIV4, treated with 50 pM RAP-103 at DIV6 and percentage of neurons with rods (shown as fold change relative to control) analyzed at DIV7. Data represent mean±SEM (n=2 replicates/treatment with ≥100 neurons/replicate). *p<0.05 by Student's *t* test.

Cofilin activation mediates αSyn-induced rod formation and dendritic spine impairment

Cofilin-actin rod formation results from the local activation of cofilin by dephosphorylation at the Ser3 residue. To test the impact of αSyn on the activation status of cofilin, we performed western blot analysis of the levels of cofilin pS3 and total cofilin on cell extracts from DIV14 cultured hippocampal neurons infected either with WT αSyn-IRES-GFP or IRES-GFP lentivirus. We observed that αSyn overexpression induced a significant decrease in the levels of cofilin pS3 with no alterations in the levels of total cofilin (Figure 3A and B). Taking into consideration that αSyn induced rods in approximately 20% of the transduced neurons, it was surprising that we detected a global decrease in cofilin phosphorylation by western blot. In the case of Aβ treatment, changes in cofilin phosphorylation were not detected by western blot as those were shown to occur only in rod-forming neurites (Walsh et al., 2014). Our data demonstrates that αSyn induces a global cofilin activation by dephosphorylation in hippocampal neurons. Having established evidence for cofilin activation in the αSyn overexpressing neurons, and since cofilin has key roles in regulating actin dynamics in neurons, we assessed the impact of cofilin activation induced by αSyn on hippocampal neuronal function. Neuronal functionality is highly dependent on the synaptic activity which includes dendritic spine integrity. There are two requirements to study dendritic spines in culture, firstly, mature neurons with developed dendritic spines, and secondly, sparse neuronal labeling to be possible to identify single dendritic processes and to analyze the respective dendritic spines. To achieve this, we cultured hippocampal neurons for 14 days and overexpressed αSyn through transfection and not transduction, as transfection allows an efficiency of 20-30% contrary to the ~100% efficiency that we observe with lentiviral infection. Initially, we evaluated rod formation in

DIV14 neurons transfected at DIV12 with the lentiviral plasmids expressing either WT αSyn-IRES-GFP or IRES-GFP. We determined a 1.8-fold increase in rod index in cells transfected with the WT αSyn-IRES-GFP plasmid when compared to cells transfected with IRES-GFP control plasmid (Figure 3C and D). To validate that αSyn-induced rod formation was a result of cofilin activation, we performed co-transfection with a cofilin phospho-mimetic inactive mutant (cofilin-S3E). Whereas cofilin-S3E had no effect on rod index in control cells, expression of the cofilin mutant totally abolished αSyn-induced rod formation (Figure 3C and D), confirming that cofilin activation induced by αSyn mediates rod formation. To further characterize the impact of αSyn-induced cofilin pathology on neurons, we assessed neuronal function focusing on dendritic spine density and morphology. Dendritic spines are small protrusions from the dendritic shaft and their integrity is critical for neuronal function, with cofilin being one of the key players in the process of spine formation and maintenance (Borovac et al., 2018). Using our neuronal culture system (Figure 3C and D), we found that αSyn-expressing neurons presented a significant decrease in dendritic spine density when compared with control neurons (Figure 3E and F). The observed reduction in dendritic spines resulted mainly from a decrease in mushroom spines, which are the most mature spine type, and filopodium spines, which represent the most dynamic and immature spine type (Figure 3E and G). This suggests that αSyn and consequent cofilin pathology affects not only spine maturation and maintenance but can also impact in stages of spine formation. Remarkably, the alterations in spine density and morphology induced by αSyn were significantly reverted, although not to control levels, when cofilin-S3E was co-expressed with αSyn (Figure 3E-G), unraveling cofilin activation as a novel player on αSyn-induced dendritic spine impairment in hippocampal neurons.

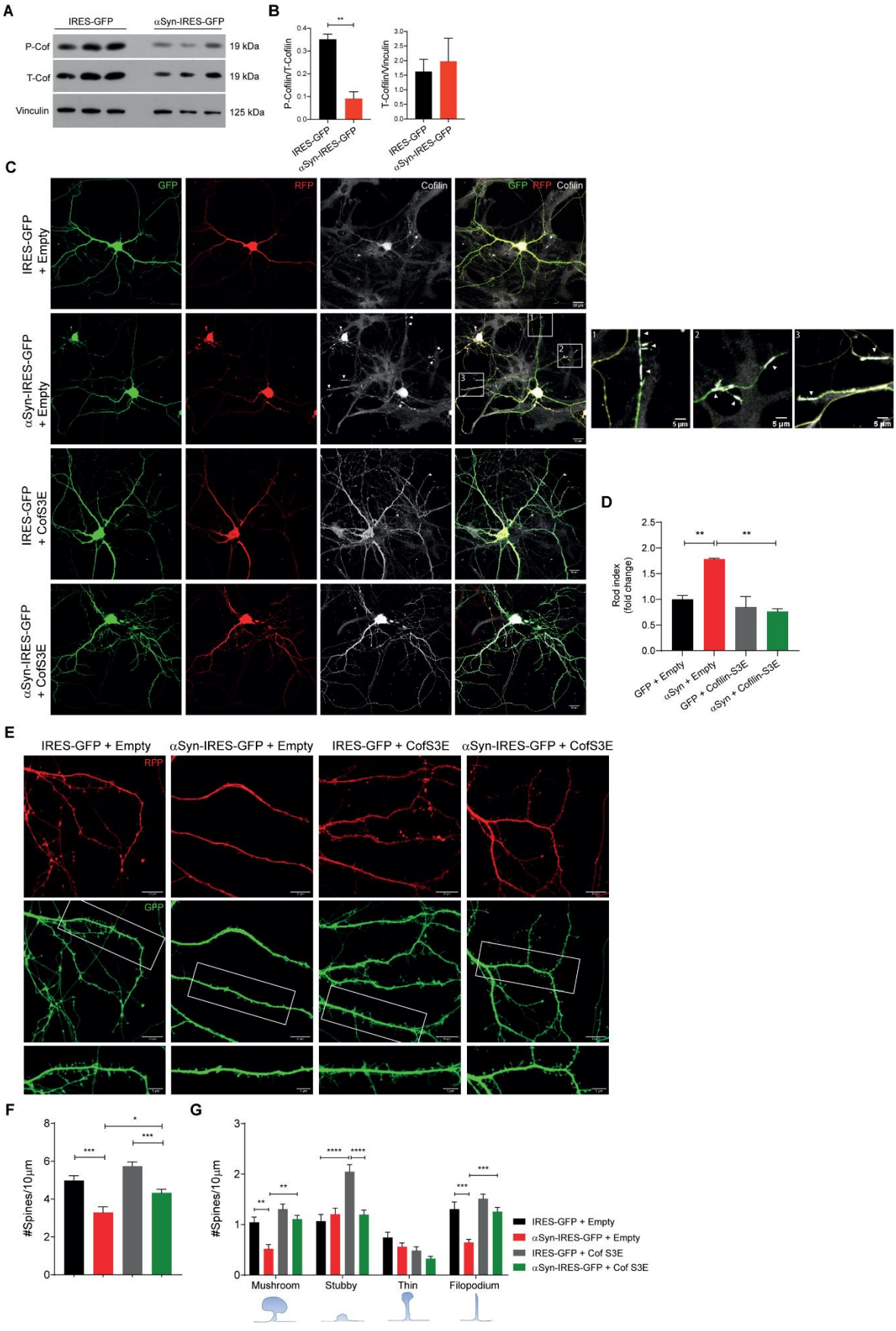


Figure 3 - Cofilin pathology underlies αSyn-induced rod formation and dendritic spine impairment. (A, B) Western blot analysis **(A)** and respective quantification by densitometry **(B)** of

cofilin pS3 and total cofilin levels in DIV14 transduced hippocampal neurons. Vinculin was used as loading control. Data represent mean±SEM (n=3 replicates/treatment). **p<0.01 by Student's *t* test. **(C)** Representative images of primary hippocampal neurons transfected at DIV12 with IRES-GFP or WT αSyn-IRES-GFP plasmids either with empty (pmRFP-N1) or cofilin-S3E (pmRFP-N1-cofilin-S3E) plasmids, and immunostained at DIV14 with cofilin (white). Scale bar: 20 μm. Insets (1, 2, 3) represent zoom-ins of rod-enriched regions and arrowheads indicate cofilin-actin rod structures. Scale bar: 5 μm. **(D)** Rod index (fold change relative to control) relative to C. Data represent mean±SEM (n=2-3 replicates/treatment with 17-50 neurons/replicate). *p<0.05 by One-way ANOVA with Tukey's multiple comparison test. **(E)** Representative images of primary hippocampal neurons transfected at DIV12 with IRES-GFP or WT αSyn-IRES-GFP plasmids either with empty (pmRFP-N1) or cofilin-S3E (pmRFP-N1-cofilin-S3E) plasmids and visualized with GFP and RFP signals at DIV14. Scale bar: 10 μm. Insets represent the respective zoom-ins noted by the white box. Scale bar: 5 μm. **(F)** Dendritic spine density relative to E. Data represent mean±SEM (n=13-25 dendrites/treatment). *p<0.05, ***p<0.001 by One-way ANOVA with Tukey's multiple comparison test. **(G)** Dendritic spine density by morphology relative to E. Data represent mean±SEM (n=13-25 dendrites/treatment). **p<0.01, ***p<0.001, ****p<0.0001 by Two-way ANOVA with Tukey's multiple comparison test.

Cofilin hippocampal pathology is observed in a mouse model of synucleinopathy with cognitive decline

Our *in vitro* data revealed that αSyn overexpression leads to an hyperactivation of cofilin in primary cultures of hippocampal neurons which results in cofilin-actin rod formation and dendritic spine impairment. We next aimed to determine whether αSyn-induced hippocampal cofilin pathology is recapitulated *in vivo*. We used a mouse model overexpressing human WT αSyn under the control of the neuronal Thy-1 promoter (Thy1-aSyn mice) (Chesselet et al., 2012). This model recapitulates the αSyn levels observed in patients with multiplications of the *SNCA* gene and presents synaptic and memory impairments starting at early stages, similarly to what is seen in PD patients who develop dementia. Using 6-months old animals, an age where synaptic and cognitive dysfunction in Thy1-aSyn was characterized (Ferreira et al., 2017), we initially confirmed the overexpression of human αSyn in the hippocampus, as well as the presence of its pathologic-associated form αSyn pS129, by immunostaining and western blot (Figure S4A-C). Additionally, we also documented animals' weight, as this mouse line was described to display a slower body weight gain when compared with WT littermates (Cuvelier et al., 2018). We observed a significant decrease in body weight, on average from 42,7 g to 31,6 g, at 6-months of age in Thy1-aSyn mice when compared with control WT littermates (Figure S4D).

We followed by validating cognitive impairment in our Thy1-aSyn mice colony. The open field test (OFT) was primarily performed to evaluate anxiety-like behaviors and locomotion as well as to habituate the animals to the arena used in the subsequent test (Figure 4A). While the total distance traveled in the open field arena was not different

between control and Thy1-aSyn (Figure 4B and D), revealing no locomotor defects, we observed that the percentage of distance traveled in the central sector of the box was decreased in the Thy1-aSyn mice (Figure 4C and D), suggesting that Thy1-aSyn mice present an anxiety-like behavior as previously described (Chesselet et al., 2012). Next, we evaluated hippocampal-related cognitive functions namely non-spatial and spatial memories tested by Normal Object Recognition (NOR) and Morris Water Maze (MWM) tests, respectively. In the NOR test (Figure 4E), the total time of exploration of the objects was not different between the two experimental groups (Figure 4F). However, whereas WT mice spent more time exploring the new object, the Thy1-aSyn mice did not distinguish between the familiar and new objects, as measured by the decreased discrimination index (Figure 4G). These results validate the decreased recognition memory which was previously reported for the Thy1-aSyn mice (Chesselet et al., 2012). In the MWM test (Figure 4H), Thy1-aSyn presented defects already in the first days of the learning phase showing increased latency to find the platform when compared with WT animals (Figure 4I). The WT control animals did not show a major improvement during the learning phase, as expected, since pretraining (cued learning) might have reduced both the stress levels and the relative deficit in spatial learning expected in the first sessions of the learning phase (Figure S4E). In the probe test, although Thy1-aSyn mice travelled the same distance as WT littermates (Figure 4J and O), they swam less distance on the target area (Figure 4K and O), travelled an increased distance to reach the target (Figure 4L and O), had an increased latency in the first entry to target (Figure 4M and O) and made fewer target crossings (Figure 4N and O). Although the distance spent in the target quadrant and the mean distance to target were not significantly different, they had a tendency to decrease and increase, respectively, in the Thy1-aSyn mice (Figure S4F and G). Together, these results confirm that Thy1-aSyn mice show impaired learning and reference memory.

Having a model of validated hippocampal αSyn pathology and cognitive impairment, provided an excellent tool to validate *in vivo* our initial hypothesis that αSyn-induced cofilin pathology plays a role on hippocampal synaptic dysfunction and cognitive deficits in LB dementias. As such, we analyzed the activation state of cofilin in the hippocampus from the 6-months old Thy1-aSyn mice. Western blot analysis revealed decreased levels of cofilin pS3 in the Thy1-aSyn mice with no alterations in the total form of the protein, validating *in vivo* αSyn-induced activation of cofilin in the hippocampus (Figure 4P and Q). Moreover, cofilin activation was accompanied by a 2.2-fold increase in rod formation in the hippocampus of Thy1-aSyn mice (Figure 4R and S), validating our *in vitro* data. We followed by analyzing whether cofilin activation was accompanied by synaptic dysfunction in Thy1-aSyn mice, by measuring the levels of the presynaptic protein VGLUT1 and the post-

synaptic protein PSD-95 in hippocampal protein extracts. Western blot results showed decreased levels of both synaptic proteins in Thy1-aSyn mice when compared with WT controls (Figure 4T-X). Interestingly, the decreased levels of PSD-95 corroborate the decrease in dendritic spine density observed *in vitro,* both cases in response to high levels of αSyn.

Our *in vivo* data shows a correlation between hippocampal cofilin pathology, alteration in the levels of synaptic proteins and cognitive dysfunction in LB dementias but does not show a definitive cause and effect relationship. Further work will address the impact of modulating cofilin activity *in vivo* in rescuing synaptic impairment and cognitive deficits in Thy1-aSyn mice.

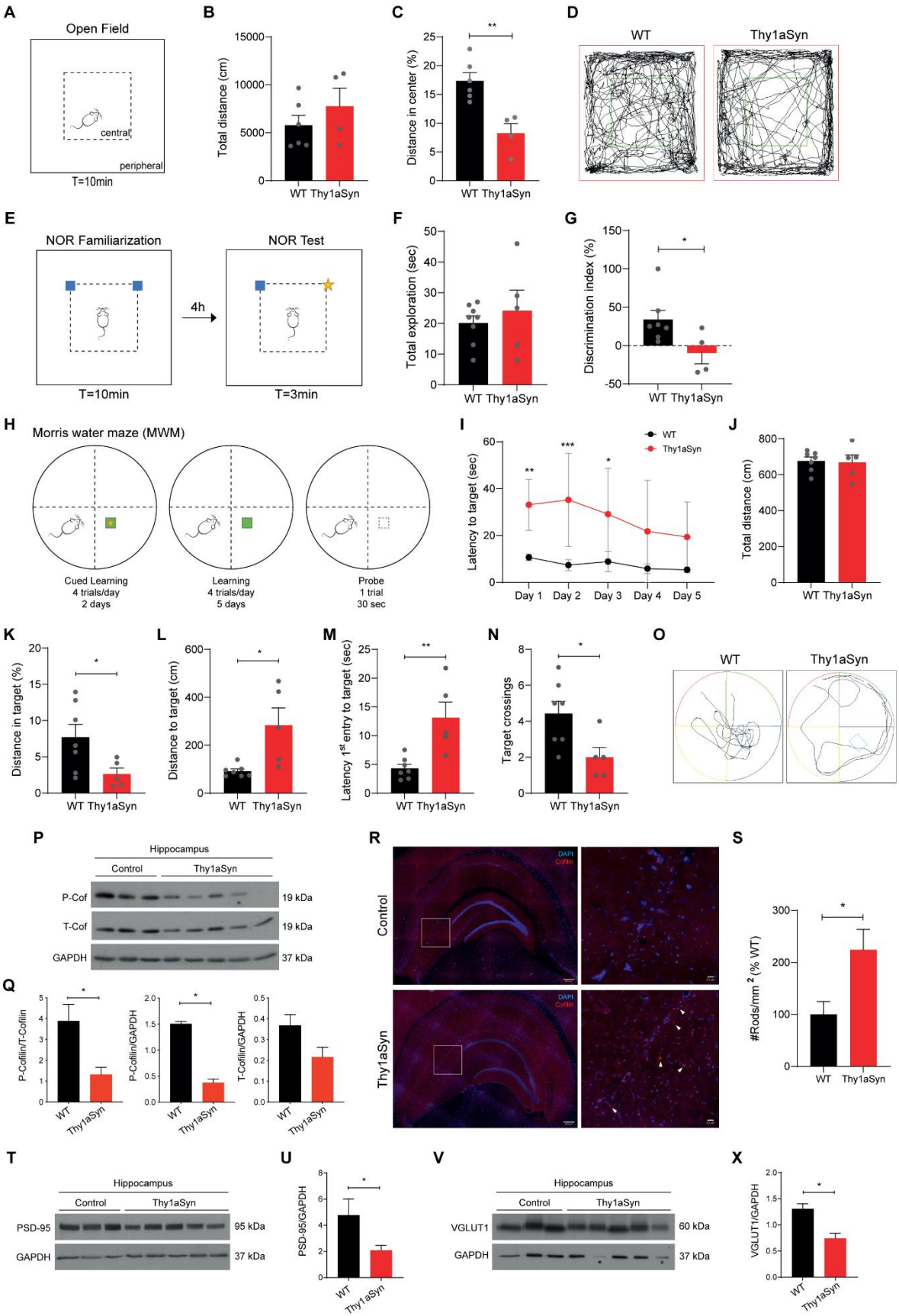


Figure 4 – Cofilin hippocampal pathology and synaptic alterations are observed in the cognitively impaired Thy1-aSyn mice. (A-D) Open field test (OFT) in 6-months old mice. **(A)**

Schematic representation of the open field arena with the central area denoted in dashed line. **(B)** Total distance traveled during the 10 min of the OFT. Data represent mean±SEM (n=4-7 animals/condition). **(C)** Percentage of distance spent in the central area of the arena. Data represent mean±SEM (n=4-6 animals/condition). **p<0.01 by Student's *t* test. **(D)** Representative tracks of WT and Thy1-aSyn mice on the OFT. **(E-G)** Novel object recognition (NOR) test in 6-months old animals. **(E)** Schematic representation of the NOR test. **(F)** Total exploration time of the familiar and new objects in the NOR test. Data represent mean±SEM (n=5-8 animals/condition). **(G)** Percentage of discrimination index in the NOR test. Data represent mean±SEM (n=4-7 animals/condition). *p<0.05 by Student's *t* test. **(H-O)** Morris Water Maze (MWM) test in 6-months old mice. **(H)** Schematic representation of the MWM test. **(I)** Latency to target in the learning phase. Data represent mean±SEM (n=5-7 animals/condition). *p<0.05, **p<0.01, ***p<0.001 by Student's *t* test. **(J-O)** Probe trial with analyses of the total distance **(J)**, distance in target **(K)**, distance to target **(L)**, latency for the 1st entry to target **(M)**, target crossings **(N)** and a representative track of the WT and Thy1-aSyn mice during the probe test **(O)**. Data represent mean±SEM (n=5-7 animals/condition). *p<0.05, **p<0.01 by Student's *t* test. **(P, Q)** Western blot analysis **(P)** and respective quantification by densitometry **(Q)** of cofilin pS3 and total cofilin levels in hippocampus from control and Thy1-aSyn mice. GAPDH was used as loading control. Data represent mean±SEM (n=3-5 animals/condition). *p<0.05 by Student's *t* test. **(R)** Representative images of brain sections from control and Thy1-aSyn mice at 6-months of age and stained for DAPI (blue) and cofilin (red) and the respective insets in the hippocampal region. Scale bar: 200 μm and 20 μm for insets. Arrowheads indicate cofilin-actin rod structures. **(S)** Number of rods per mm^2 (percentage relative to WT) in the hippocampus region relative to R. Data represent mean±SEM (n=4-5 animals/condition with 2-3 sections/animal). *p<0.05 by Student's *t* test. **(T, U)** Western blot analysis **(T)** and respective quantification by densitometry **(U)** of PSD-95 levels in hippocampus from control and Thy1-aSyn mice with 6-months of age. GAPDH was used as loading control. Data represent mean±SEM (n=3-5 animals/condition). *p<0.05 by Student's *t* test. **(V, X)** Western blot analysis **(V)** and respective quantification by densitometry **(X)** of VGLUT1 levels in hippocampus from control and Thy1-aSyn mice with 6-months of age. GAPDH was used as loading control. For the purpose of quantification, the two animals with very low levels of GAPDH* were not considered. Data represent mean±SEM (n=3 animals/condition). *p<0.05 by Student's *t* test.

Discussion

Cognitive dysfunction and dementia symptomatology critically affects functioning and quality of life of patients with LB dementias. These symptoms have been related to αSyn hippocampal pathology, as αSyn-containing inclusions are regularly detected in the hippocampus of patients with synucleinopathies, which present hippocampal atrophy and dysfunction as well as cognitive deficits (Yang and Yu, 2017). Nevertheless, the specific molecular mechanisms underlying hippocampal dysfunction need further investigation, being important to determine the pathologic consequences of αSyn accumulation in that brain region. The major findings of this work demonstrated both *in vitro* and *in vivo* that αSyn overexpression in the rodent hippocampus induces cofilin activation by dephosphorylation and cofilin-actin rods formation. In αSyn-overexpressing primary cultures of hippocampal neurons, cofilin activation partially mediated dendritic spine impairment. Importantly, and in accordance with our *in vitro* data, we correlated hippocampal cofilin pathology triggered by αSyn with synaptic dysfunction and cognitive impairment *in vivo* by using the Thy1-aSyn mice, a suitable model to study cognitive deficits in the context of LB dementias (Hatami and Chesselet, 2015).

Cofilin dysregulation has been previously associated to αSyn pathology in primary cultures of hippocampal neurons. Studies by Chieregatti and colleagues reported an αSyn-induced activation of the actin signaling pathway Rac1/PAK2/LIMK/cofilin-1 *via* GRP78 in hippocampal neurons resulting on cofilin phosphorylation and inactivation, and consequent blockage of actin dynamics (Sousa et al., 2009; Bellani et al., 2014). Here we present an opposite observation with αSyn inducing cofilin activation in hippocampal neurons. This discrepancy might be related with distinct experimental conditions of αSyn exposure. While in the reported study there was an acute addition of 1μM recombinant αSyn to DIV14 neurons (Bellani et al., 2014), in our settings hippocampal neurons were transduced at DIV4 and analyzed at DIV14 where we observed a 3-fold increase in αSyn levels. Additionally, we validated cofilin activation *in vivo* in the hippocampus of Thy1-aSyn mice with overexpression of αSyn. These overexpression scenarios are more suitable for our propose of studying hippocampal αSyn pathology in the context of LB dementias as they recapitulate αSyn levels detected in patients with multiplications of the *SCNA* gene, which often develop cognitive deficits. More importantly, the Thy1-aSyn mouse model is one of the few models that show cognitive deficits at an early age, what makes it particularly suitable to study the molecular mechanisms in a premanifest phase of the disease as well as to proceed with preclinical drug studies. Nevertheless, it would be interesting to further analyze the activation status of cofilin in additional mouse models and also focus on other cell types

such as dopaminergic neurons, the ones mainly affected in typical motor PD, to determine whether αSyn-induced cofilin activation has neuronal subtype specificity.

Subsequently to cofilin activation induced by αSyn overexpression, we observed the formation of cofilin-actin rods in hippocampal neurons, which were reverted by using the phosphomimetic mutant cofilin-S3E, confirming the need of cofilin activation for rod formation. Corroborating our findings, in a mouse model of AD, the genetic decrease in cofilin expression or the administration of cofilin-pS3 peptide led to a decrease in cofilin activity and positively impacted in synaptic function and cognitive behavior (Woo et al., 2015b; Deng et al., 2016). Although not related to hippocampal pathology, cofilin-actin rod formation was previously reported in a *Drosophila* model of αSyn overexpression, and in the cingulate cortex of patients with neocortical Lewy Body pathology (Ordonez et al., 2018).

We observed neuronal rod formation induced not only by αSyn overexpression but also in response to the exogenous addition of soluble forms of αSyn. Focusing on the data with mature DIV14 neurons, which reflect a more physiologic scenario, it might suggest that the intraneuronal accumulation of αSyn pS129 has a major contribution to rod formation, as shown in the experiments of long-term αSyn overexpression. Nevertheless, in those experiments, αSyn was also present in the extracellular milieu, what combined with the observations showing that short-term addition of αSyn released from neurons or αSyn recombinant species resulted in rod formation, suggest an extracellular αSyn-interaction with a membrane receptor. In this respect, in our settings, αSyn-induced rods was mediated by PrP^C and chemokine receptor CCR5 pathways, similarly to what was reported for Aβ and more recently for the HIV gp120 envelope protein (Walsh et al., 2014; Smith et al., 2021).

Rod formation induced by cofilin overexpression was shown to impair synaptic function in hippocampal neurons (Cichon et al., 2012), what was validated in our model by showing that cofilin pathology is involved in spine impairment induced by αSyn. Remarkably, αSyn pathophysiology is a stimuli that occurs *in vivo*, contrary to cofilin overexpression, what reinforces the relevance of our findings. Cofilin pathology might have consequences to synaptic function through either physical blockage of vesicle transport or sequestration of cofilin, depleting it from synaptic structures and impacting in actin dynamics and function of these structures (Cichon et al., 2012; Rust, 2015). Interestingly, PrP^C was previously described to interact with αSyn, mediating hippocampal synaptic impairment and cognitive deficits in PD (Ferreira et al., 2017). Considering the inhibitory effect of PrP^C knockdown on αSyn-induced rod formation, our data includes cofilin pathology as a new intermediate on the mechanism of αSyn-PrP^C induction of synaptic and cognitive dysfunction.

Interestingly, we found an additional player in αSyn-induced cofilin pathology, the CCR5 chemokine receptor. Interestingly, CCR5 has been increasingly implicated in neurodegenerative disorders including AD and PD (Choi et al., 2013; Li and Zhu, 2019). In particular, a study showed that the oral administration of maraviroc, a CCR5 inhibitor, in hemiparkinsonian monkeys resulted in reduced infiltration of T cells into the substantia nigra, decreased glial activation, lowered αSyn pathology and promoted dopaminergic neurons protection resulting in improved motor function (Mondal et al., 2019). These findings support the idea of the involvement of chemokine receptors in the development of PD, which may also be involved in LB dementias. In our studies we used RAP-103, which is an orally available and shorter analog of DAPTA (D-alapeptide T-amide) that was one of the first HIV gp120-derived CCR5 inhibitors (Padi et al., 2012). RAP-103 was previously described to inhibit CCR2/CCR5 in human monocytes and its oral administration contributed to the prevention and attenuation of neuropathic pain symptoms in rats (Padi et al., 2012). Additionally, in mouse models of cortical stroke and traumatic brain injury, where loss of neuronal functions was observed, CCR5 was found upregulated and after its knockdown or pharmacologic inhibition, mice revealed improved motor and cognitive function what might be derived from the preservation of dendritic spines (Joy et al., 2019). Corroborating a role for CCR5 in cofilin activity, the use of CCR5 inhibitors prevented cofilin-actin rod formation in gp120-treated neurons (Smith et al., 2021). Further supporting an involvement of CCR5 in cofilin activity, some studies have shown cofilin activation upon HIV gp120 binding to chemokine receptors in blood resting CD4 T cells during infection (Yoder et al., 2008). In fact, cofilin activation was shown to be required for HIV latent infection of blood resting CD4 T cells. During infection, HIV binding to chemokine receptors triggers cycles of cofilin activation and inactivation, leading to increased cortical actin dynamics facilitating virus entry (Yoder et al., 2008; Vorster et al., 2011; He et al., 2019; He and Wu, 2019). Together, these studies support a role for the involvement of cofilin activation upon CCR5 activation, what further supports our findings.

Taking all this into consideration, it is conceivable a role for chemokine receptors in cofilin pathology and synaptic dysfunction, of which PrP^C may serve as a co-receptor, and we propose that RAP-103 constitutes a promising therapeutic approach, not only for LB dementias but also for AD or HAND, where similar molecular mechanisms were described. Overall, our results strengthen the link between αSyn-induced hippocampal synaptic dysfunction and cognitive deficits in LB dementias and identify cofilin as a novel pathologic player.

Future work will address the effect of targeting cofilin pathology in the Thy1-aSyn mice, to validate our hypothesis that cofilin dysregulation is responsible for synaptic

dysfunction in the context of dementia in synucleinopathies. Current treatments for LB dementias include mainly the use of cholinesterase inhibitors, as cholinergic deficit in patients with dementia was reported. However, efficacy and safety of cholinesterase inhibitors needs further analysis in larger clinical trials (Wang et al., 2015; Meng et al., 2019). Moreover, PD treatments mainly focus on increasing neurotransmitter signaling and it is important to develop new pharmacological approaches to prevent or reverse synapse loss and neuronal connectivity. As such, the development of new therapies targeting dementia in the context of synucleinopathies is imperative, and our future goal of targeting cofilin pathology, which impacts on neuronal dysfunction, constitutes a promising innovative strategy impacting not only on LB dementias but also in additional disorders, namely AD and HAND, where rods were reported.

Materials and Methods

Animals

Animal procedures were performed in accordance with national and European rules. The protocols described in this work have been approved by the IBMC Ethical Committee and by the Portuguese Veterinarian Board and by the Institutional Animal Care and Use Committee of Colorado State University (protocols KP1023 and KP1412). Transgenic mice overexpressing human αSyn under the neuronal Thy-1 promoter (Thy1-aSyn mice, Line 61 developed on a C57Bl6/DBA2 background) (Rockenstein et al., 2002) were kindly provided by University of California San Diego. The colony background was maintained by breeding mutant females with WT C57BL/6-DBA/2 males. Since the transgene insertion was on the X chromosome which is randomly inactivated in females, only male littermates were used in the experiments.

PrP^C null mice (PrP-/-) TALEN (Nuvolone et al., 2016) were generously provided by Mark Zabel, Prion Research Center, Colorado State University.

Time pregnant wild-type female Wistar rats (E18) or C57BL/6 mice (E16.5) were used for the dissociated neuronal cultures.

Plasmids and viral vectors

IRES-GFP and WT αSyn-IRES-GFP lentiviral plasmids were previously described (Paiva et al., 2018). Briefly, full-length human WT αSyn c-DNA was subcloned into the pWPI vector (second generation bicistronic lentiviral vector, Tronolab, Switzerland), under the chicken/β-actin (CBA) promoter. A pWPI vector containing only IRES-GFP was used as control. pmRFP-N1 and pmRFP-N1-Cofilin-S3E plasmids were previously described (Garvalov et al., 2007). Briefly, pseudo-phosphorylated human cofilin-1 (S3E) was cloned into the pmRFP-N1 backbone vector, under the CMV promoter. An empty pmRFP-N1 vector was used as control.

Lentiviruses production and titration

Lentivirus production was performed as previously described (Naldini et al., 1996). Briefly, HEK293T cells were transfected, using Lipofectamine 2000 (ThermoFisher Scientific, 11668030), with the DNA complexes containing the plasmid of interest and the packaging plasmids (psPAX2 and VSV-G), for 5 h at 37ºC/5%CO_2. After 5 h of incubation, medium was replaced with DMEM (VWR, 733-1695) supplemented with 10% FBS (Biowest, BWSTS181BH-500) and 1% penicillin/streptomycin (P/S) (ThermoFisher Scientific, 15140-122). Following 48 h, the lentivirus-containing supernatants were recovered, centrifuged for

10 min at 500 g and filtered using a 0.45 μm filter (Enzifarma). The filtered supernatants were concentrated using a centricon (GE Healthcare Life Sciences), aliquoted and stored at -80ºC. For virus titration, HEK293T cells were infected with different lentivirus dilutions. After 3 days, cells were resuspended in PBS and the total number of transduced cells was analyzed by Flow Cytometry using FACS Accuri (BD Biosciences). The lentiviral transduction units (TU) per μL were determined by the following equation: TU/μL= (number of plated cells x % of infected cells (GFP positive)) / volume of viral particles added (μL).

αSyn monomers and oligomers

αSyn monomers and oligomers were prepared from recombinant human αSyn as previously described (Diogenes et al., 2012). Prepared species were aliquoted and stored at -80°C and thawed in ice immediately before use.

Dissociated hippocampal neuron cultures, transduction and drug treatments

The hippocampus was dissected from E18 rat embryos (WT) or E16.5 mouse embryos (WT and PrP^{C} KO), digested with 0.06% trypsin (Sigma-Aldrich, T4799) in Hanks' balanced salt solution (HBSS, Sigma, H9394) for 15 min at 37ºC. Following digestion, neurons were dissociated by gentle trituration and resuspended in neurobasal medium (Invitrogen, 21103049) supplemented with 2% N21-MAX (R&D Systems, AR008), 1% P/S and 2.5 mM L-Glutamine (Lonza, 17-605E). Cells were then counted and platted at a density of 15,000 cells/coverslip in a 24-well plate for immunostaining analysis, or at a density of 200,000 cells/well in a 6-well plate for western blot analysis. Coverslips and plates were precoated with 20 μg/mL poly-D-lysine (Sigma, P0899).

For neuronal transduction, DIV 3 hippocampal neurons were treated with 1 μM of (+)-MK-801 hydrogen maleate (Sigma, M107) for 30 min at 37ºC to reduce spontaneous rod formation. At DIV4, neurons were infected either with WT αSyn-IRES-GFP or IRES-GFP lentiviruses (1TU/cell). At DIV7 or DIV14 culture medium was recovered and cells were fixed or lysed for cell extracts.

For RAP-103 treatment, DIV13 hippocampal neurons transduced with WT αSyn-IRES-GFP or IRES-GFP lentiviruses were treated with RAP-103 (CCR5 antagonist, Creative BioPeptides, Inc.) at 50 pM diluted in water. Hippocampal neurons were analyzed at DIV7 or DIV14, 24 h after treatment with the antagonist.

Hippocampal neurons transfection

DIV 3 hippocampal neurons were treated with 1 μM of (+)-MK-801 hydrogen maleate (Sigma, M107) for 30 min at 37ºC to reduce spontaneous rod formation. At DIV12 neurons were transfected using the calcium phosphate co-precipitation method with the constructs: αSyn-IRES-GFP, IRES-GFP, pmRFP-N1-Cofilin-S3E, or pmRFP-N1. Briefly, a maximum amount of 2 μg of DNA was diluted in Tris-EDTA (TE) pH 7.3 and mixed with HEPES calcium chloride (2.5 M CaCl2 in 10 mM of HEPES pH7.2). This mixture was added to 2x HEBS (270 mM NaCl, 10 mM KCl, 1.4 mM Na2HPO4, 11 mM Dextrose, 42 mM HEPES pH7.2) and the precipitate was allowed to develop during 30 min at RT in the dark, with gentle mixing every 5 min. Culture medium was removed and saved while neurons were incubated with plain neurobasal medium and the precipitates were added dropwise to each well. Precipitates were incubated with cells for 45 min at 37ºC/5%CO_2. Precipitate solution was then removed and neurons washed with acidic neurobasal medium (equilibrated at 10% CO_2) for 20 min at 37ºC/5%CO_2. Lastly, the medium was replaced with the saved supplemented medium. Neurons were analyzed at DIV14, 48 h after transfection.

Hippocampal slice cultures and transduction

Hippocampal slice cultures were performed as previously described (Fixman et al., 2017). Briefly, 4-7 days old mouse pups were euthanized and the brain collected. The hippocampus was then dissected and chopped in 300 μm slices. Coverslips were prepared with 2 μL of chicken plasma and each slice was placed in the spot containing the plasma. A mixture of 1:1 of plasma with thrombin was added and within few seconds the plasma clot was formed and the slice attached to the coverslip. Slices were flushed with 5% CO_2/95% air mixture and cultured in a 35°C roller incubator with complete medium containing: Neurobasal A, 2% N21-MAX, 2.5 mM L-Glutamine, 0.1% Glucose and 1% penicillin/streptomycin. Medium was replaced on day 3 or 4 and again on day 7.

For slice transduction, hippocampal slices were treated with IRES-GFP and WT αSyn-IRES-GFP lentiviral plasmids at DIV9 and fixed for analysis at DIV15.

Dot blot

Supernatants collected from transduced hippocampal neurons were centrifuged at 4000 g for 5 min at 4ºC to remove cell debris and then applied to a nitrocellulose membrane using FisherbrandTM Dot Blot Hybridisation Manifold System according to the manufacturer's recommended protocol. Membranes were dried and then rehydrated and blocked for 1 h at RT with 5% milk/TBS-T and incubated overnight at 4ºC with the primary antibody mouse monoclonal anti-αSyn 1:1000 (BD Biosciences, 610787) in 5% milk/TBS-

T. After washing, membranes were incubated 1 h at RT with secondary antibody anti-mouse IgG-HRP diluted in 5% milk in TBS-T. Immunodetection was performed by chemiluminescence using ECL reagent.

Immunocytochemistry

For immunostaining, cells were fixed with 4% PFA in cytoskeleton preservation buffer (10 mM 2-(N-morpholino) ethanesulfonic acid (MES, Sigma-Aldrich, M3671) pH 6.1; 3 mM $MgCl_2$ (Merk, 1.05833.0250); 138 mM KCl (Merk, 529552); 2 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA, Sigma-Aldrich, E8145); 0.32 M sucrose (Merk, 1.07651.1000)), for 30 min at room temperature (RT). For cofilin-actin rods staining, neurons were permeabilized with cold (-20ºC) 100% methanol for 3 min at RT and blocked with 2.5% normal serum from donkey (Jackson ImmunoReasearch, 017-000-121) or goat (Sigma, 19H092) in 1% BSA/PBS for 1 h at RT. Next, cells were incubated with primary antibodies: rabbit anti-T-cofilin 1:2000 (Bamburg lab, 1439 or Cell Signalling, 5175) and mouse anti-β3-tubulin 1:2000 (Promega, G7121) diluted in 1% BSA/PBS and incubated overnight at 4ºC. After washing, cells were incubated with secondary antibodies: donkey anti-mouse-Alexa Fluor 488 1:1000 (Invitrogen, A21202) or donkey anti-mouse-Alexa Fluor 647 1:1000 (Invitrogen, A31571) and donkey anti-rabbit–Alexa Fluor 568 1:1000 (Invitrogen, A10042) diluted in 1% BSA/PBS. Coverslips were mounted in Fluoromount-G (SouthernBiotech, 0100-01) or ProLong Diamond Antifade (ThermoFisher P36961). For αSyn staining, neurons were permeabilized with 2.5% triton X-100 in PBS for 20 min at RT and blocked with 5% normal donkey serum in 1% BSA/PBS for 1 h at RT. Subsequently, neurons were incubated with primary antibodies: mouse anti-β3-tubulin 1:2000 or mouse anti-αSyn 1:1000 (BD Biosciences, 610787) and rabbit anti αSyn pS129 1:1000 (Abcam, ab51253) diluted in 1% BSA/PBS and incubated overnight at 4ºC. After washing and incubation with secondary antibodies: donkey anti-rabbit–Alexa Fluor 568 1:1000 (Invitrogen, A10042) and donkey anti-mouse-Alexa Fluor 647 1:1000 (Invitrogen, A31571) diluted in 1% BSA/PBS, coverslips were mounted in Fluoromount-G.

Immunohistochemistry

For immunostaining analysis, mice were perfused with PBS for 5 min followed by 4% paraformaldehyde (PFA, pH 7.4) in PBS (40 mL). Brains were incubated in 4% PFA for 24h and then in 30% sucrose. Brain tissues were embedded in Optimum Cutting Temperature (OCT) compound (ThermoFisher Scientific), frozen and sectioned coronally (Cryostat Leica CM3050S) at 30 µm. Cultured hippocampal slices were fixed with 4% PFA in cytoskeleton preservation buffer (described above). For cofilin-actin rods staining brain sections and

hippocampal slices were permeabilized with 100% methanol at -20ºC for 5 min at RT and blocked with 5% normal donkey serum in PBS for 1 h at RT, followed by incubation overnight at 4ºC with primary antibody rabbit anti-T-cofilin 1:1000, and subsequent washing and incubation with secondary antibody donkey anti-rabbit–Alexa Fluor 568 1:500 diluted in 1% BSA/PBS. Brain sections were then washed and rinsed in 70% ethanol and incubated with Sudan Black in 70% ethanol for 10 min at RT. Sections and slices were washed and mounted in Ibidi mounting medium (Ibidi, 50001).

For αSyn pS129 immunostaining, brain sections were washed with 0.3% triton X-100 in PBS and blocked with 1% normal donkey serum in 0.3% triton X-100 in PBS for 1 h at RT. Next, brain sections were incubated with primary antibody rabbit anti-αSyn pS129 1:5000. Subsequently, sections were washed and incubated with secondary antibody donkey anti-rabbit-Alexa Fluor 568 1:1000, washed, and mounted in Ibidi mounting medium.

Western blot

For western blot analysis mice were perfused with PBS for 5 min, and the hippocampus was dissected and quickly frozen in dry ice. Frozen brains were incubated with RIPA buffer (1% Triton X-100, 0.1% SDS, 140 mM NaCl, 1x TE pH 8, 1x protease inhibitor Cocktail and 1 mM sodium orthovanadate), sonicated (2x10 cycles, Output Power 50 Watts, Branson sonifier 250) and cleared by centrifugation at 15000 rpm for 5 min at 4ºC. For western blot of cultured neurons, cells were harvested and lysed in 0.3% triton X-100 (Sigma-Aldrich, T9284), 1x protease inhibitor Cocktail (100x, GE Healthcare, 80-6501-23) and 1 mM sodium orthovanadate (Sigma-Aldrich, S6508). 25 μg or 5 μg of protein extracts were separated under denaturing conditions in 12% SDS-PAGE gels and transferred to nitrocellulose membranes (0.45 μm GE HealthCare) for 2 h, using a semi-dry transfer system (CBS scientific EBU-4000) or a wet transfer system (Bio-rad, 1703930). Membranes were blocked with 5% milk (Sigma-Aldrich) in TBS-T or 5% BSA (NZYTech) in TBS-T for 1 h at RT. Membranes were probed overnight at 4ºC with the following primary antibodies: mouse anti-αSyn 1:1000 (BD Biosciences, 610787), rabbit anti-αSyn pS129 1:500 (Abcam, ab51253), rabbit anti-T-cofilin 1:1000 (Bamburg lab, 1439 or Cell Signaling, 5175), mouse anti-T-cofilin 1:500 (Abcam, ab54532), rabbit anti-P-cofilin Ser3 1:1000 (Cell Signaling, 3311), mouse anti-PSD-95 1:2000 (ThermoFisher Scientific, MA1-046), rabbit anti-VGLUT1 1:5000 (Synaptic Systems, 135303), rabbit anti-vinculin 3:10000 (ThermoFisher Scientific, 700062) and mouse anti-GAPDH 1:1000 (Santa Cruz, sc-166574) diluted in 5% milk/TBS-T or 5% BSA. After washing, the membrane was incubated for 1 h at RT with secondary antibodies: anti-mouse IgG-HRP 1:10000 (Jackson Research, 115-035-003) or anti-rabbit IgG-HRP 1:10000 (Jackson Research, 111-035-003) diluted in

5% milk/TBS-T. Immunodetection was performed by chemiluminescence using ECL (Millipore, WBLUR0500). Quantitative analyses were performed with the Quantity One software or Fiji software.

Imaging and quantifications

DIV7 hippocampal neurons immunostained for cofilin were assessed for the presence of cofilin-actin rods in an upright epifluorescence microscope (Zeiss Axio Imager Z1, Carl Zeiss) at 40x magnification. The percentage of neurons with cofilin-actin rods was plotted.

DIV14 hippocampal neurons immunostained for cofilin were imaged in an automated fluorescence widefield high-content screening microscope (IN Cell Analyzer 2000, GE Healthcare) at 40x magnification. Images were analyzed using Fiji software and the ratio between the number of rods and the total number of neurons analyzed was calculated and plotted as Rod index.

For spine density quantification, DIV14 hippocampal neurons expressing GFP, were imaged in a laser scanning Confocal Microscope Leica SP5 AOBS SE, using the 63x oil objective. Dendritic length and spine number and morphology were quantified using the semi-automatic NeuronStudio software (Rodriguez et al., 2006). Dendritic spine morphology was defined as mushroom spines (small neck and large head), thin spines (long neck and small head), stubby spines (head without a defined neck) and filopodium spines (long neck without defined head). Spine density was defined by the number of dendritic spines by 10 μm of dendritic length.

Hippocampal slices were imaged in a laser scanning Confocal Microscope Leica SP5 AOBS SE, using the 40x water objective. 6 fields of each region were acquired and the number of rods in each condition quantified and plotted.

Brain sections stained for cofilin were imaged in an automated fluorescence widefield high-content screening microscope (IN Cell Analyzer 2000, GE Healthcare) at 20x magnification. Images were stitched and the brain regions of interest analyzed using Fiji software and the results plotted as number of rods per area.

Behavioral tests

Animals were used in behavioral tests at 6-months of age.

Open-field (OF)

WT and Thy1-aSyn mice were subjected to the OF test during the dark period to evaluate activity levels. The system comprised an empty opaque squared arena (40x40 cm). Individually, mice were placed in the center of the arena and left to explore the area

for 10 min. The box was cleaned between animals to eliminate odor or traces from the previous animal. Mice behavior parameters, as total distance travelled and activity in the central and peripheric areas, were recorded and posteriorly analyzed by the software The Smart v3.0, Panlab, Barcelona, Spain.

Novel object recognition (NOR)

Animals that have been previously tested in the OF (habituation) were subsequently subjected to the NOR test to evaluate memory. In the same arena used in the OF, animals were exposed to two identical objects for 10 min for familiarization. In the test phase, four hours later, one of the familiar objects was replaced by a new object from the same material, weight and height, but with a different color and shape, and the animal was allowed to explore for 3 min. Exploration was considered when the mouse's nose touched the object or when the nose is directed to the object from a distance less than 2 cm. Mouse behaviors were recorded and analyzed by the software The Observer XT v7.0, Noldus, Netherlands. The discrimination index was calculated by DI = (TN − TF)/(TN + TF) in which TN is the time exploring the novel object and TF is the time spent exploring the familiar object. Animals with a total exploration time (novel + familiar) less than 10 seconds were excluded from the analysis of the discrimination index (Bevins and Besheer, 2006).

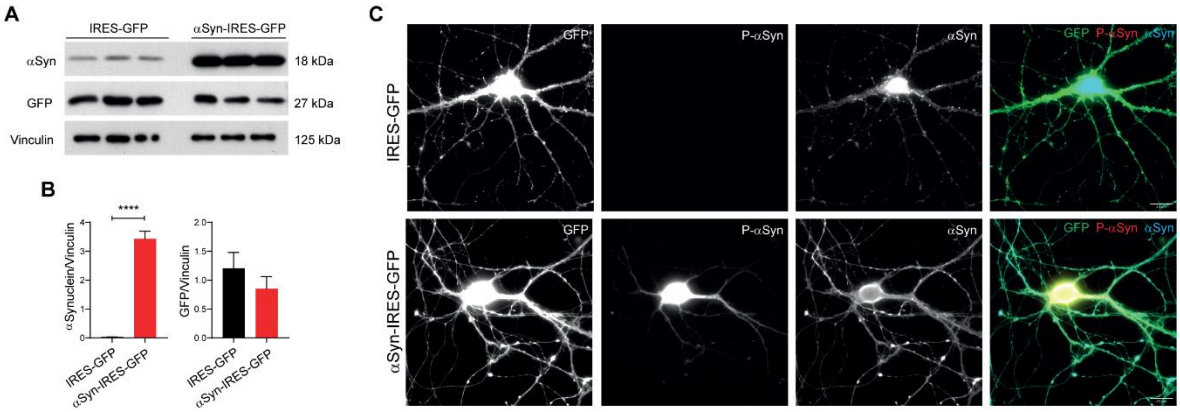
Morris water maze (MWM)

Spatial learning was assessed by the hidden-platform Morris water maze test. A circular pool (diameter 111 cm) was used and filled with water at 21±1ºC. The pool was theoretically divided in four quadrants and eight start positions were defined at equal distance to the center. An escape platform (10x10 cm) was placed 0.5 cm below the water line. In first two days, during the cued leaning, mice were trained to find the hidden platform which had a visual clue. Animals were subjected to four swimming trials which had a different start and goal position. In the next 5 days, during the learning phase, mice were trained to find the hidden platform which is in the same location during the entire learning phase. Every day mice were subjected to four swimming trials, each trial starting at one of the four different pool locations, and the latency to find the platform was scored. If mice failed to find the platform within 1 min they were guided to the platform. In either case, mice stayed on the platform for 15 sec. On the eighth day, in the probe day, the platform was removed from the pool and the mice swam for 30 sec. Swimming tracks were recorded and analysis of the total distance, distance to and in target, latency for the 1st entry to target and target crossings were obtained with The Smart v3.0, Panlab, Barcelona, Spain.

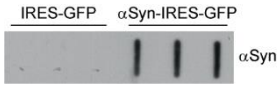
Statistical analysis

All measurements were performed with the researcher blinded to the experimental condition when possible. Data are shown as mean ±SEM (standard error of the mean). Statistical significance was determined using the GraphPad Prism Software version 8 and the most appropriate statistic test, being significance determined by *p<0.05, **p<0.01, **p<0.001 and ****p<0.0001. Statistic test and sample sizes are indicated in each figure legend.

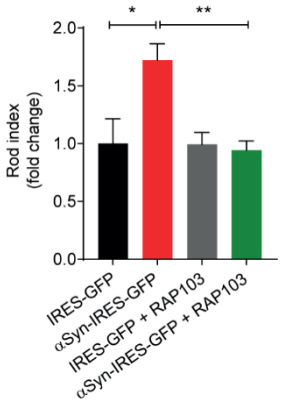
Supplementary Figures



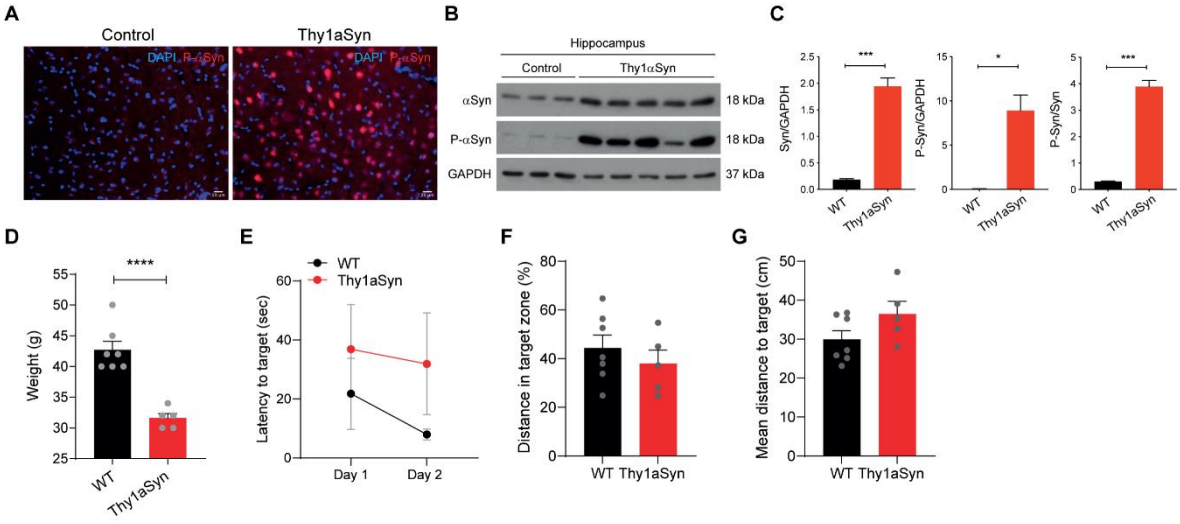
Supplementary Figure S1 - Overexpression of αSyn in mature hippocampal neurons. (A, B) Western blot analysis **(A)** and respective quantification by densitometry **(B)** of αSyn and GFP levels in DIV14 transduced hippocampal neurons with IRES-GFP or WT αSyn-IRES-GFP lentivirus at DIV4. Vinculin was used as loading control. Data represent mean±SEM (n=3 replicates/treatment). ****p<0.0001 by Student's *t* test. **(C)** Representative images of DIV14 transduced hippocampal neurons immunostained with αSyn pS129 (red) and αSyn (blue). Scale bar: 20 μm.



Supplementary Figure S2 - αSyn is released from transduced hippocampal neurons in culture. Dot blot analysis of αSyn levels in the supernatants from DIV7 IRES-GFP or WT αSyn-IRES-GFP transduced neurons.



Supplementary Figure S3 - CCR5 is involved in αSyn-induced rod formation in mature neurons. Primary hippocampal neurons were infected with IRES-GFP or WT αSyn-IRES-GFP lentivirus at DIV4, treated with 50 pM RAP-103 at DIV13 and rod index (fold change relative to control) analyzed at DIV14. Data represent mean±SEM (n=2-3 replicates/treatment with 40-100 neurons/replicate). *p<0.05, **p<0.01 by Student's *t* test.



Supplementary Figure S4 - Thy1-aSyn mice show increased αSyn pS129 in hippocampus and cognitive defects. (A) Representative images of brain sections from 6-months control and Thy1-aSyn mice stained for DAPI (blue) and αSyn pS129 (red). Scale bar: 20 μm. **(B, C)** Western blot analysis **(B)** and respective quantification by densitometry **(C)** of αSyn and αSyn pS129 levels in hippocampus from control and Thy1-aSyn mice. GAPDH was used as loading control. Data represent mean±SEM (n=3-5 animals/condition). *p<0.05, ***p<0.001 by Student's *t* test. **(D)** Weight of 6-months control and Thy1-aSyn mice. Data represent mean±SEM (n=5-7 animals/condition). ****p<0.0001 by Student's *t* test. **(E-G)** Behavioral analysis of 6-months old mice in MWM test. **(E)** Latency to target during cued learning. **(F)** Distance in target zone. **(G)** Mean distance to target. Data represent mean±SEM (n=5-7 animals/condition).

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CHAPTER II

α-Synuclein pre-formed fibrils induce cofilin-actin rods formation in hippocampal neurons *via* PrP^C-NOX and CCR5/CXCR4-dependent pathways

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Abstract

α-Synuclein (αSyn) is a pre-synaptic protein which dysregulation and aggregation constitute the major hallmark of synucleinopathies. According to Lewy Body (LB) pathology staging, αSyn pathology spreads in a stereotypic manner starting in discrete regions and propagating to wider brain regions. Growing evidences have demonstrated that αSyn fibrillary forms can be transmitted from cell-to-cell where they act as a template promoting the aggregation of the endogenous protein. Additionally, it has been reported that hippocampal neurons treated with αSyn pre-formed fibrils (αSyn PFFs), which develop αSyn pathology, show decreased synaptic function which ultimately culminates in neuronal death. Nevertheless, the underlying molecular mechanisms of αSyn PFFs-induced pathology to the hippocampal region and the correlation with the subjacent cognitive impairment are still understudied. To tackle this question, we used recombinant αSyn PFFs, *in vitro,* using dissociated hippocampal neurons, and *in vivo,* using a mouse model of αSyn PFFs injection, and evaluated cofilin-actin rods formation as we have previously reported rod formation upon αSyn overexpression (Chapter I). We found that αSyn PFFs induce the formation of cofilin-actin rods, through signaling pathways involving the cellular prion protein (PrP^C)-NADPH oxidase (NOX) and the chemokine receptors CCR5/CXCR4. Interestingly, we also show that a long-term incubation with αSyn PFFs or a combined strategy of αSyn PFFs addition to hippocampal neurons overexpressing αSyn achieved high levels of rod response, supporting a contribution of αSyn seeding, and most likely spreading, for hippocampal cofilin pathology. Additionally, an *in vivo* mouse model of αSyn PFFs injection showed rod formation in the hippocampal region. Remarkably, our data indicates cofilin dysregulation as a novel player in αSyn PFFs-induced hippocampal pathology, which may also play a role in the progression of hippocampal synaptic dysfunction.

Introduction

αSynuclein (αSyn) neuronal inclusions are the pathologic hallmark of synucleinopathies which includes Lewy Body (LB) dementias, characterized by cognitive impairment (Albert et al., 1974). αSyn aggregation in LBs occurs intracellularly, however, the finding that αSyn is present in the extracellular fluids including the blood plasma and CSF in both physiologic and pathologic conditions, opened a new field of research regarding synucleinopathies (Borghi et al., 2000; El-Agnaf et al., 2003). In addition to this, Braak staging proposes that αSyn pathology is transmitted between neurons leading to the spreading of the pathology between interconnected regions of the nervous system (Braak et al., 2004). This idea, together with the findings of αSyn-positive LB pathology in the grafted neurons of PD patients several years after the transplantation, raised the hypothesis of a prion-like propagation of αSyn, in which αSyn pathologic species self-propagate to the neighbor neurons seeding the aggregation of the endogenous protein (Kordower et al., 2008; Li et al., 2008). This neuron-to-neuron αSyn propagation has been increasingly established in the literature as the underlying mechanism by which αSyn pathology propagates through the brain. In order to study cell-to-cell αSyn spreading and seeding, several studies have been using recombinant synthetized αSyn pre-formed fibrils (αSyn PFFs) because they form LB-like structures that closely resemble those found in patients (Volpicelli-Daley et al., 2014). αSyn PFFs spread in a prion-like fashion, implying that they are spread between cells and seed the aggregation of the endogenous protein in the recipient cells, and this was demonstrated to occur *in vitro* and *in vivo* (Luk et al., 2009; Luk et al., 2012a). Additionally, an attractive feature of using these models relies on the fact that they are more suitable to recapitulate sporadic disease, as the αSyn PFFs constitute the initial trigger for αSyn aggregation but the levels of endogenous protein are unaltered. Importantly, it has been shown that long-term incubation with αSyn PFFs compromise synaptic function in a time- and dose-dependent manner in hippocampal neurons (Wu et al., 2019). More specifically, these studies reported that αSyn PFFs induced a decrease in synaptic proteins, altered dendritic spines and impairment of neuronal excitability, which positively correlated with increased αSyn pathology, and ultimately led to neuronal death (Volpicelli-Daley et al., 2011; Wu et al., 2019). Additional studies, in which αSyn PFFs were administered *in vivo* in the striatum, showed the detrimental effects of αSyn seeding for the dendritic architecture and for dendritic spine density in cortical regions (Blumenstock et al., 2017).

These models constitute a promising strategy to study disease pathogenesis, however, the molecular mechanisms underlying the αSyn PFFs impact on hippocampal

synaptic function requires further investigation. Considering this, and that we have previously observed that exogenous addition of αSyn-containing supernatants or αSyn monomers and oligomers induce cofilin-actin rod formation in hippocampal neurons (Chapter I), we questioned whether αSyn PFFs might also trigger cofilin dysregulation and rod formation, which may impact in hippocampal synaptic function in these models. Cofilin-actin rod structures were previously reported in the context of AD, in ischemic stroke and more recently upon exposure to the viral protein gp120 (Minamide et al., 2000; Rahman et al., 2014; Shu et al., 2018; Won et al., 2018; Smith et al., 2021). Rods are formed upon localized hyperactivation of cofilin and in a reactive oxygen species (ROS)-enriched environment and were shown to occlude intracellular trafficking and promote spine loss (Cichon et al., 2012).

Here we show that inoculation of hippocampal neurons with αSyn PFFs triggers the formation of cofilin-actin rods *in vitro* and *in vivo*, *via* a molecular pathway involving PrP^{C}-NOX and chemokine receptors CCR5 and CXCR4 in hippocampal neurons. In summary, our results suggest cofilin as a new player in the mechanism of αSyn PFFs-induced hippocampal pathology, which constitute a good candidate by which αSyn modulates synaptic activity.

Results

Cofilin-actin rod formation is triggered in a model of exogenous addition of αSyn PFFs

We have previously demonstrated that αSyn-overexpressing hippocampal neurons release soluble αSyn that induces cofilin-actin rods in non-αSyn-transduced neurons (Chapter I). Additionally, further experiments showed that αSyn monomers and oligomer species were also able to promote cofilin-actin rod formation in WT neurons (Chapter I). These results suggest that αSyn spreading might play a role in hippocampal cofilin pathology. In addition, several reports have shown that the exogenous addition of αSyn PFFs, derived from the aggregation of recombinant αSyn monomers, is a suitable model promoting formation of αSyn pathogenic inclusions in WT neurons, as well as being involved in αSyn spreading and synaptic dysfunction in hippocampal neurons (Wu et al., 2019). Therefore, we aimed to also analyze rod formation in a model of exogenous addition of αSyn PFFs. Initially, and considering an extracellular effect of the exogenous addition of αSyn on rod formation, we questioned whether a 24 h addition of αSyn PFFs to hippocampal neurons could induce rod formation. We observed that treatment of DIV6 WT rat hippocampal neurons with 1 µg/mL of αSyn PFFs induced a 2.7-fold increase in the percentage of neurons with rods at DIV 7 (Figure 1A and B).

Together, these findings indicate that exogenous αSyn PFFs trigger cofilin-actin rods formation in hippocampal neurons.

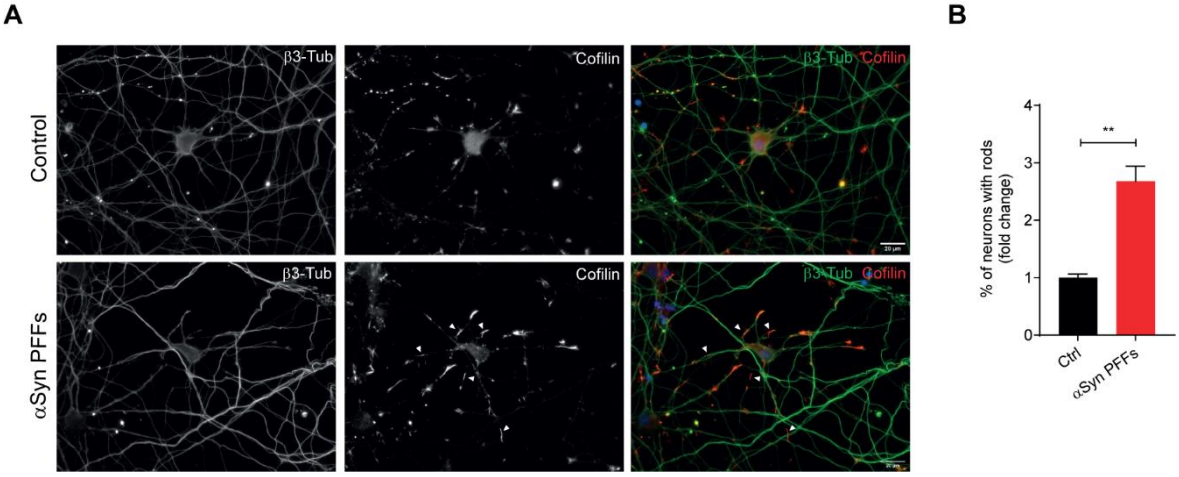

Figure 1 - Cofilin-actin rods are formed upon αSyn PFFs treatment in hippocampal neurons. **(A)** Representative images of DIV7 hippocampal neurons pre-treated with control (PBS) or αSyn PFFs (1 µg/mL) for 24 h and immunostained for β3-tubulin (green) and cofilin (red). Scale bar: 20 µm. Arrowheads indicate rod structures. **(B)** Percentage of neurons with rods (shown as fold change relative to control) relative to A. Data represent mean±SEM (n=3 replicates/treatment with ≥100 neurons/condition). **p<0.01 by Student's *t* test.

αSyn PFFs seeding potentiate cofilin-actin rod formation

In order to analyze the impact of αSyn PFFs spreading and seeding in rod formation, in a more pathophysiologic context, we performed analysis of DIV14 neurons, when endogenous αSyn is detectable and synapses are mature. Moreover, to potentiate the seeding process, some reports have combined an overexpression system with the addition of αSyn PFFs (Thakur et al., 2017). Considering this, we infected hippocampal neurons with either WT αSyn-IRES-GFP or IRES-GFP lentivirus at DIV4, treated them at DIV7 with 150 ng/mL of αSyn PFFs and analyzed rod formation at DIV14. As we previously demonstrated (Chapter I), αSyn-overexpression induced rod formation in mature neurons as observed by the 1.7-fold increase in rod index (Figure 2A and B). αSyn PFFs induced a 2.9-fold increase in rod index in GFP-transduced cells while in αSyn-transduced neurons the increment in rod formation was of approximately 3.5-fold, when compared to αSyn-transduced neurons, and a remarkable 5.2-fold when compared with control condition (IRES-GFP) (Figure 2A and B). In this experiment we detected αSyn pS129 by immunocytochemistry in αSyn-transduced neurons and in GFP-transduced neurons treated with αSyn PFFs, being the highest levels of αSyn pS129 observed in WT αSyn-transduced neurons with αSyn PFFs (Figure 2C). These findings support that the process of seeding, and probably of spreading, triggered by αSyn PFFs, resulting in intracellular accumulation of αSyn pS129, potentiates αSyn-induced rod formation.

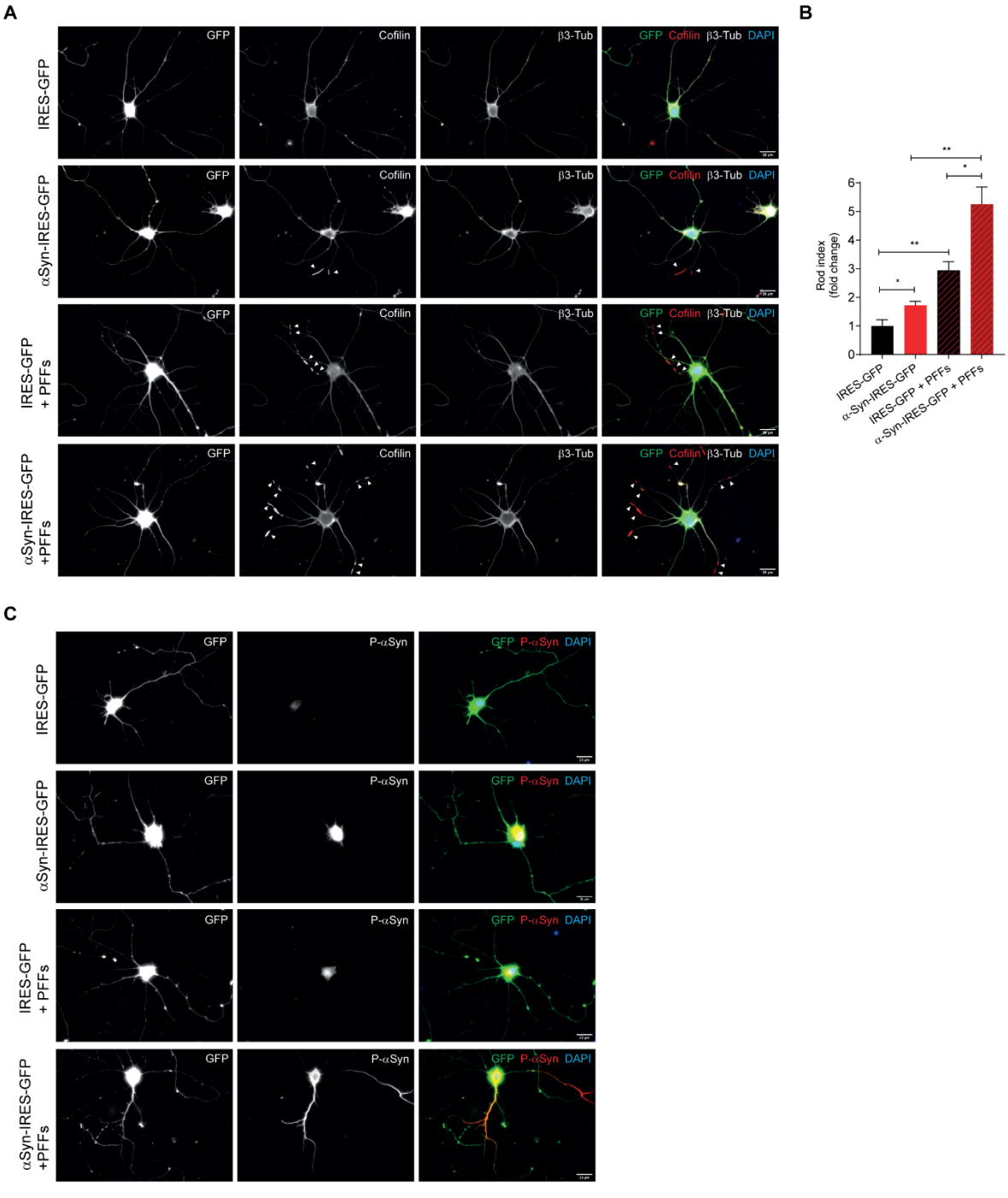

Figure 2- αSyn PFFs seeding potentiate cofilin-actin rod formation. (A) Representative images of hippocampal neurons infected with IRES-GFP or WT αSyn-IRES-GFP lentivirus at DIV4 with or without addition of αSyn PFFs (150 ng/mL) at DIV7 and immunostained at DIV14 for β3-tubulin (white) and cofilin (red). Scale bar: 20 μm. Arrowheads indicate rod structures. **(B)** Rod index (Number of rods/number of neurons, shown as fold change relative to control) relative to A. Data represent mean±SEM (n=3 replicates/treatment with ≥100 neurons/condition). *p<0.05, **p<0.01 by Student's *t* test. **(C)** Representative images of hippocampal neurons treated with IRES-GFP or WT αSyn-IRES-GFP lentivirus at DIV4 with or without addition of αSyn PFFs (150 ng/mL) at DIV7 and immunostained at DIV14 for αSyn pS129 (red). Scale bar: 20 μm.

PrP^C-NOX and CCR5/CXCR4 are involved in rod formation induced by αSyn PFFs

Having established a model of αSyn PFFs-induced rod formation, we aimed at dissecting the molecular mechanism underlying this effect. As αSyn PFFs exert an effect in a short-term incubation, we hypothesized their interaction with a membrane receptor triggering a signaling cascade. PrP^C-NOX pathway was shown to mediate Aβ-induced rod formation in hippocampal neurons (Walsh et al., 2014), and we have also previously showed that PrP^C was involved in rod response induced by αSyn overexpression (Chapter I). Interestingly, PrP^C was reported to be important for the transfer of αSyn between cells leading to spreading of pathology (Aulic et al., 2017). More recently, it was demonstrated that PrP^C interaction with αSyn existent in extracellular milieu, triggers a signaling pathway that culminates in synaptic dysfunction in hippocampal neurons (Ferreira et al., 2017). Taking this into consideration, we treated hippocampal neurons from WT and PrP^C KO mice at DIV6 with 1 µg/mL of αSyn PFFs and analyzed them at DIV7. We observed, by the percentage of neurons with rods, that αSyn PFFs induced a 3.1-fold increase in rod formation which was abolished when using PrP^C KO neurons (Figure 3A). Additionally, to test the involvement of NOX in αSyn induced rod formation, we used neurons from p47 KO mice, which is an essential subunit of NOX, and observed that αSyn PFFs were not able to promote rod formation (Figure 3A). These findings confirm the involvement of PrP^C-NOX pathway in αSyn PFFs-induced cofilin pathology.

We have previously demonstrated that the chemokine receptor CCR5 was involved in rod response triggered by αSyn overexpression (Chapter I). Additionally, a recent report showed that the use of chemokine receptor antagonists, such as maraviroc or AMD3100, which target CCR5 and CXCR4, respectively, significantly reduced the number of rods in neurons in response to gp120, an HIV-derived envelop protein, and upon $Aβ_{d/t}$ treatment (Smith et al., 2021). Taking this into consideration, we hypothesized that exogenous αSyn might contribute to cofilin hippocampal pathology *via* chemokine receptors, which include not only CCR5, but also the chemokine receptor CXCR4. To address this hypothesis, we cultured hippocampal neurons and exposed them at DIV6 with αSyn PFFs alone or in the presence of the CXCR4 antagonist AMD3100, or the CCR5 antagonist maraviroc, or an antagonist of both receptors, RAP-310, and analyzed the neurons at DIV7. While we observed a 3.9-fold increase in the percentage of neurons with rods in αSyn PFFs-treated neurons, the use of chemokine receptor antagonists completely abolished this effect (Figure 3B). This further proposes a common molecular mechanism between Aβ and αSyn in inducing hippocampal pathology.

These findings point to an extracellular effect of αSyn PFFs on rod induction, which seems not to be related with seeding and intracellular aggregation, as on one hand, DIV7 neurons do not show detectable levels of endogenous protein, and on the other hand, immunocytochemistry analysis of DIV7 neurons pre-treated with αSyn PFFs for 24 h did not detect αSyn pS129 (data not shown), as previously described (Volpicelli-Daley et al., 2014; Wu et al., 2019). Further experiments should test the efficacy of chemokine receptors inhibitors in rescuing rod formation in mature neurons with long-term treatment of αSyn PFFs, to clarify whether the pathways are similar for short- and long-term incubations.

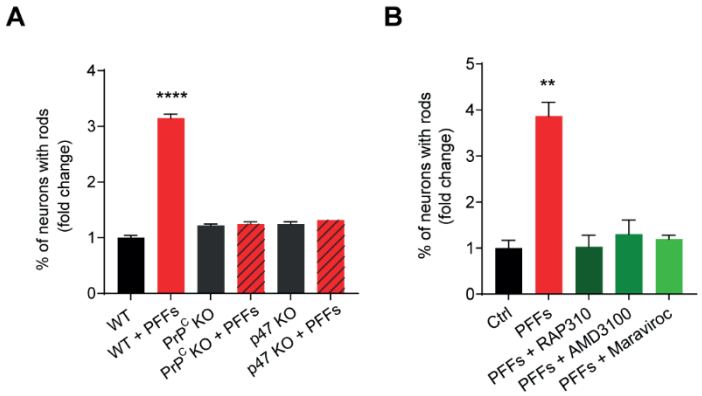

Figure 3 - αSyn PFFs induce cofilin-actin rods formation *via* PrP^C-NOX and CCR5/CXCR4. (A) Quantification of the percentage of neurons with rods (shown as fold change relative to control) in DIV7 hippocampal neurons from WT, PrP^C KO or p47 KO mice pre-treated for 24 h with control (PBS) or αSyn PFFs 1 μg/mL. Data represent mean±SEM (n=3 replicates/treatment with ≥100 neurons/replicate). ****p<0.0001 refers to αSyn PFFs versus all the other conditions, by Two-way ANOVA with Tukey's multiple comparison test. **(B)** Hippocampal neurons were treated with control (PBS) or αSyn PFFs 1 μg/ml only or with the addition of RAP-310 (10 pM), Maraviroc (60 nM), or AMD3100 (60 nM) at DIV6 and the percentage of neurons with rods was analyzed at DIV7. Data represent mean±SEM (n=2 conditions/treatment with ≥100 neurons/condition). **p<0.01 refers to αSyn PFFs versus all the other conditions, by Two-way ANOVA with Tukey's multiple comparison test.

αSyn PFFs-injection in vivo trigger cofilin-actin rods in the hippocampus

Aiming at assessing whether αSyn PFFs have an effect on rod formation *in vivo*, we used a mouse model of αSyn PFFs injection in the substantia nigra. This model was chosen to analyze the effect of spreading *in vivo*, as αSyn species injected in the substantia nigra pars compacta triggered the aggregation of the endogenous protein in the cortex, striatum, thalamus, and hippocampus (Aulic et al., 2017). We observed the formation of cofilin-actin rods in the hippocampus of mice 7-months post-injection, which presented a 2.9-fold increase in rod formation compared to control injected mice (Figure 4A and B). These results recapitulate our *in vitro* data of long-term incubation of αSyn PFFs which induced rod formation in hippocampal neurons. More importantly, these findings suggest that

spreading, and probably seeding, of αSyn species contributes to cofilin pathology. Previous studies using a mouse model of αSyn PFFs-injection in the striatum, and analyzed 5-months after injection, showed αSyn aggregation concurrent with dendritic architecture impairment as well as spine loss in cortical regions (Blumenstock et al., 2017). Our findings propose cofilin dysregulation as a novel player in the mechanism of αSyn PFFs-induced hippocampal pathology. Future studies with this model of PFFs injection should address cofilin phosphorylation levels in the hippocampal region, as well as hippocampal-related spine integrity and cognitive function, in order to correlate hippocampal cofilin pathology with synaptic dysregulation and cognitive impairment.

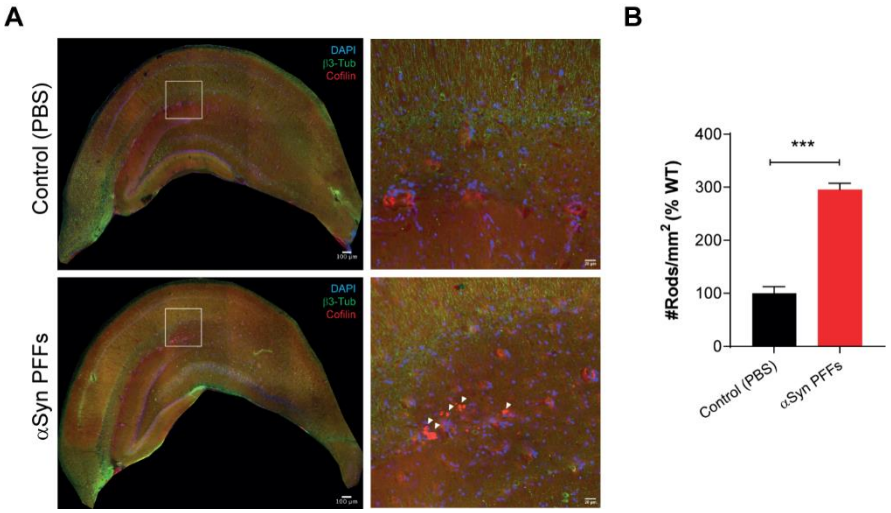

Figure 4 – *In vivo* administration of αSyn PFFs leads to hippocampal rod formation. (A) Representative images of brain sections from control (PBS) and αSyn PFFs injected WT mice, 7-months after the injection. Sections stained for DAPI (blue), β3-tubulin (green) and cofilin (red) and the respective insets in the hippocampal region. Scale bar: 100 μm and 20 μm for insets. Arrowheads indicate cofilin-actin rod structures. **(B)** Number of rods per mm^2 (percentage relative to WT) in the hippocampal region relative to A. Data represent mean±SEM (n=3-4 animals/condition with 3 sections/animal). ***p<0.001 by Student's *t* test.

Discussion

The spreading of αSyn pathology is considered to contribute to the cell-to-cell transmission of the aggregated forms of αSyn which serve as seeds to convert the intracellular protein into pathologic aggregates in the recipient cells (Braak et al., 2004; Kordower et al., 2008; Li et al., 2008). This process of αSyn spreading and seeding contributes to the propagation of pathology throughout several brain regions contributing to disease progression. Several aspects of the disease are recapitulated using *in vitro* and *in vivo* models with αSyn PFFs (Chung et al., 2020). Nevertheless, the exact molecular mechanisms underlying αSyn PFFs-induced neuronal pathology remain unclear, and we proposed to further investigate these in hippocampal neurons. Here we demonstrate that exogenous αSyn PFFs induce cofilin-actin rods in hippocampal neurons and this effect involves the PrP^C-NOX pathway and the chemokine receptors CCR5 and CXCR4. This is consistent with our previous findings showing the disturbance of the actin binding protein cofilin and the formation of cofilin-actin rods, upon αSyn overexpression (Chapter I). Moreover, the same molecular pathways were found to mediate Aβ- and gp120-induced rod formation (Smith et al., 2021) as well as rod formation triggered by αSyn overexpression (Chapter I). Reactive oxygen species (ROS) production is a common outcome triggered by several neurodegenerative stimuli which invariably lead to rod formation, and in our settings we observed that using p47 KO neurons, which is a cytosolic subunit of NOX without which NOX complex formation is not possible (Babior, 2004), αSyn PFFs no longer exert an effect in rod formation. PrP^C was described to mediate synaptic dysfunction in hippocampal neurons upon αSyn stimuli (Ferreira et al., 2017), which suggests that rods might be an additional player in αSyn-PrP^C-induced synaptic dysfunction, as we observed a blockage of rod formation in neurons from PrP^C KO mice. PrP^C is a broad co-receptor indicating that it might serve as a co-receptor for several partners as is the case of Aβ, gp120 and αSyn. Importantly we also found, by using chemokine antagonists, maraviroc, AMD3100 and RAP-310, that αSyn-induced rod formation is additionally mediated by CCR5 and CXCR4. In fact, PrP^C, NOX and chemokine receptors are proposed to be enriched in lipid raft domains, which are thought to serve as scaffolds that promote the association of signaling proteins and increase the rate of interactions (Jin et al., 2011; Lewis and Hooper, 2011), what might contribute to an enhanced crosstalk between the different signaling pathways found here to be involved in αSyn hippocampal pathology. More specifically, it is plausible that PrP^C might serve as a co-receptor facilitating αSyn interaction with chemokine receptors. Chemokine receptors have been increasingly implicated in neurodegenerative disorders such as AD and PD (Li and Zhu, 2019; Gavriel et al., 2020). Focusing on PD, a

report showed that the oral administration of maraviroc, a CCR5 antagonist, contributed to amelioration of motor function, αSyn pathology and promoted dopaminergic neurons protection in a model of hemiparkinsonism in monkeys (Choi et al., 2013). Considering the involvement of CXCR4 in PD, some reports showed an increased expression of CXCR4 and CXCL12, which is its natural ligand, in the substantia nigra of PD patients (Shimoji et al., 2009; Li et al., 2019). Additionally, in two mouse models of PD, one expressing A53T αSyn mutant and other induced with MPTP, increased levels of CXCR4 and CXCL12 were also reported in the substantia nigra accompanied by microglial activation (Shimoji et al., 2009; Li et al., 2019). Although these studies majorly address PD-related motor function, they suggest the involvement of neuroinflammation, and specifically of the chemokine receptors CCR5 and CXCR4, in the etiology of synucleinopathies, and together with our findings showing αSyn-induced rod inhibition with chemokine receptors antagonists in hippocampus, these pathways likely play a role in LB dementias as well. Future work should address the impact of the use of chemokine receptor antagonists *in vivo*, in αSyn PFFs-injected animal models with the goal of developing new therapeutic avenues targeting cognitive impairment in LB dementias.

In respect to short incubation of αSyn PFFs which triggered rod formation, this was somewhat a surprising result as seeding does not occur within this time frame (Volpicelli-Daley et al., 2014). Nonetheless, there is a report showing that an acute infusion of αSyn PFFs by patch clamp decreased mEPSC (miniature excitatory postsynaptic currents) frequency within 10 min, however, dendritic spines were not altered in this context contrary to the observed with long-term incubations of αSyn PFFs (Wu et al., 2019). The acute infusion also triggered an accelerated αSyn pathology, which was attributed to a bypass of the internalization processes and a direct access to the intracellular environment (Wu et al., 2019). In any case, it was always attributed an intracellular effect to αSyn PFFs, which contrasts with our findings, since we have observed not only rod formation with short-time addition of αSyn PFFs, but also rod inhibition with receptor antagonists or genetic knockdown, implying an extracellular role for αSyn PFFs.

Neuronal rod formation was induced not only by short-term incubation with αSyn PFFs to hippocampal neurons, but also in response to the long-term exposure to αSyn PFFs, which achieved a maximum rod response in cells that also overexpressed αSyn. Focusing on these data with mature DIV14 neurons, which reflect a more pathophysiologic scenario, our findings suggest that the intraneuronal accumulation of αSyn pS129 has a significant contribution to rod formation. Nevertheless, in those experiments, we cannot exclude the presence of αSyn in the extracellular milieu, and considering our observations

with short-term addition of αSyn PFFs, this suggests a combined effect of αSyn-interaction with membrane receptors and αSyn intracellular accumulation. In fact, the so-called αSyn spreading can have distinct outcomes what corroborates our findings. In one hand, can lead to the activation of signaling cascades at the cell membrane of itself or of neighbor neurons, while in the other hand, can lead to αSyn uptake and seeding of the endogenous protein by other neurons, or both. Previous studies have reported the detrimental effect of the long-term incubation of αSyn PFFs in hippocampal neurons, which resulted in progressive accumulation of the pathologic-associated phosphorylated form of the protein, with consequences for synaptic function and dendritic spine density and morphology (Volpicelli-Daley et al., 2011; Froula et al., 2018; Wu et al., 2019). Importantly, these effects are observed before neuronal loss, which is consistent with previous reports showing that compromised synaptic activity and dendritic spine loss constitute one of the initial and predominant pathophysiological mechanism triggered by αSyn aggregates, which occurs before neuronal death (Kramer and Schulz-Schaeffer, 2007; Nikolaus et al., 2009; Froula et al., 2018). Interestingly, cofilin pathology, and specifically cofilin-actin rods were also described to impair axonal transport and synaptic functions preceding neuronal death (Cichon et al., 2012), what further supports the idea that cofilin might be an intermediate player in the αSyn PFFs-induced spine impairment and synaptic dysregulation. Supporting this, cofilin, and its correct regulation, is essential for maintaining a proper actin cytoskeleton architecture and function in dendritic spines, which is detrimental for spine morphology and consequent synaptic function (Noguchi et al., 2016). Further supporting a role for αSyn-induced cofilin pathology and neuronal dysfunction, previous studies have shown that extracellular αSyn might affect membrane permeability or lipid raft integrity which results in increased calcium influx (Pacheco et al., 2015; Emanuele et al., 2016). Considering that calcium binds calmodulin forming a complex that activates calcineurin which in turn dephosphorylates slingshot (SSH1) making it available to dephosphorylate and activate cofilin (Wang et al., 2005), it is plausible an impact of high levels of calcium, triggered by αSyn aggregates, on cofilin activation and consequent dysregulation culminating in rod formation.

Importantly, in an *in vivo* mouse model of αSyn pathology, where αSyn PFFs were injected in the substantia nigra, we observed rod formation in the hippocampal region several months after the injection. This model was previously described to have a spread αSyn pathology reaching several brain regions such as the cortex, striatum, thalamus, and hippocampus (Aulic et al., 2017). Additional studies provided evidence that αSyn PFFs injected in the striatum promote αSyn pathology in cortical regions which was accompanied by dendritic arborization disruption and spine impairment (Blumenstock et al., 2017).

Moreover, another model of αSyn spreading, determined by the injection of αSyn PFFs in the gut, which reached the brain, resulted in the development of several neuropathologic-related features as well as cognitive impairment (Kim et al., 2019). Interestingly, it has been described that while αSyn PFFs seem to be essential for the initial steps of the aggregation to occur, the pathogenic intracellular αSyn aggregates are majorly composed by endogenous protein, and only trace amounts of αSyn PFFs were detected in the inclusions (Luk et al., 2009). Since the majority of synucleinopathies are of sporadic etiology, where the levels of αSyn are expected to be relatively normal, the αSyn PFFs models, where the levels of endogenous protein are unaltered, represent a promising strategy to study the molecular mechanisms underlying sporadic disease. Considering our findings and the current knowledge, it would be important to address synaptic integrity and cognitive performance in our mouse model of αSyn PFFs injection to clarify whether hippocampal cofilin pathology induced by αSyn spreading and seeding contributes to synaptic dysfunction and cognitive decline.

Taking into account our findings, we suggest that hippocampal rod formation seems to be the result of a combined cell-autonomous and non-cell autonomous mechanism which results from intraneuronal αSyn aggregation and αSyn spreading, respectively. These conclusions were supported not only by our *in vitro* data but also by the *in vivo* data with the αSyn PFFs-injected mice. Finally, having an established system inhibiting αSyn PFFs-induced rod formation and consequent hippocampal pathology, it would be interesting to investigate to what extent the rescue in rod formation recovers synaptic function, dendritic spine morphology and density and ultimately cognitive functions, *in vivo*, with the ultimate goal of proving cofilin as a new therapeutic avenue for LB dementias.

In summary, we showed that αSyn PFFs induce cofilin-actin rods in dissociated hippocampal neurons and in the hippocampal brain region, *via* PrP^{C}-NOX and CCR5/CXCR4 pathways; mechanisms that we suggest to contribute to hippocampal synaptic dysfunction and cognitive impairment, described to exist in the αSyn PFFs models, and that may explain the synaptic dysregulation and cognitive decline present in LB dementia patients.

Materials and Methods

Animals

Animal procedures were performed in accordance with national and European rules. The protocols described in this work have been approved by the IBMC Ethical Committee and by the Portuguese Veterinarian Board, by the Institutional Animal Care and Use Committee of Colorado State University (protocols KP1023 and KP1412) and in accordance with the US PHS Policy on Humane Care and Use of Laboratory Animals and with protocols approved by the San Francisco Veterans Affairs Medical Center animal studies committee and by the Institutional Animal Care and Use Committee of Colorado State University (protocols KP1023 and KP1412).

p47 null mice (p47phox-/-) JAX stock number 027331 B6N.129S2-Ncf1tm1Shl/J; PrP^{C} null mice (PrP-/-) TALEN (Nuvolone et al., 2016); Breeding pairs of p47 null mice were generously provided by Dr. Ray Swanson, University of California, San Francisco. PrP-/- mice were generously provided by Mark Zabel, Prion Research Center, Colorado State University.

Time pregnant wild-type female Wistar rats (E18) or C57BL/6 mice (E16.5) were used for the dissociated neuronal cultures.

Pre-formed fibrils (PFFs)

For pre-formed fibrils (PFFs) preparation, human αSyn monomer protein was purchased from Proteos (RP-003). PFFs were prepared according to the protocol established by Michael J Fox Foundation for Parkinson's Research (Patterson et al., 2019). Briefly, αSyn monomers were diluted to 5 mg/ml into 0.01 M phosphate buffered saline (PBS) containing 0.03% sodium azide to prevent bacterial growth. Monomers were shaken for 7 days at 1000 rpm and 37°C in an orbital shaker to induce the formation of fibrils (PFFs). Single-use aliquots were rapidly frozen and stored at -80°C. Immediately before use, frozen aliquots were thawed, diluted in PBS to 0.1 mg/ml and bath sonicated at room temperature for 5 minutes.

Plasmids and viral vectors

IRES-GFP and WT αSyn-IRES-GFP lentiviral plasmids were previously described (Paiva et al., 2018). Briefly, full-length human WT αSyn c-DNA was subcloned into the pWPI vector (second generation bicistronic lentiviral vector, Tronolab, Switzerland), under the chicken/β-actin (CBA) promoter. A pWPI vector containing only IRES-GFP was used as control.

Lentiviruses production and titration

Lentivirus production was performed as previously described (Naldini et al., 1996). Briefly, HEK293T cells were transfected, using Lipofectamine 2000 (ThermoFisher Scientific, 11668030), with the DNA complexes containing the plasmid of interest and the packaging plasmids (psPAX2 and VSV-G), for 5 h at 37ºC/5%CO_2. After the incubation, medium was replaced with DMEM (VWR, 733-1695) supplemented with 10% FBS (Biowest, BWSTS181BH-500) and 1% P/S. After 48 h, the lentivirus-containing supernatants were recovered, centrifuged for 10 min at 500g and filtered using a 0.45 μm filter (Enzifarma). The filtered supernatants were concentrated using a centricon (GE Healthcare Life Sciences), aliquoted and stored at -80ºC. For virus titration, HEK293T cells were infected with different volumes of lentivirus. After 3 days, cells were resuspended in PBS and the total number of transduced cells was analyzed by Flow Cytometry using FACS Accuri (BD Biosciences). The lentiviral transduction units (TU) per μL were determined by the following equation: TU/μL= (number of plated cells x % of infected cells (GFP positive)) / volume of viral particles added (μL).

Dissociated hippocampal neuron cultures, transduction and drug treatments

The hippocampus was dissected from E18 rat embryos or E16.5 mouse embryos, digested with 0.06% trypsin (Sigma-Aldrich, T4799) in Hanks' balanced salt solution (HBSS, Sigma, H9394) for 15 min at 37ºC. Following digestion, neurons were dissociated by gentle trituration and resuspended in neurobasal medium (Invitrogen, 21103049) supplemented with 2% N21-MAX (R&D Systems, AR008), 1% penicillin/streptomycin (ThermoFisher Scientific, 15140-122) and 2.5 mM L-Glutamine (Lonza, 17-605E). Cells were then counted and platted at a density of 15,000 cells/coverslip in a 24-well plate for immunostaining analysis. Coverslips were precoated with 20 μg/mL poly-D-lysine (Sigma, P0899).

For hippocampal neuron transduction, DIV 3 hippocampal neurons were treated with 1 μM of (+)-MK-801 hydrogen maleate (Sigma, M107) for 30 min at 37ºC to reduce spontaneous rod formation. At DIV4, neurons were infected either with WT αSyn-IRES-GFP or IRES-GFP lentiviruses (1TU/cell). Hippocampal neurons were analyzed at DIV7 or DIV14.

For chemokine receptor antagonists treatment, DIV 6 hippocampal neurons were treated alone or in the presence of αSyn PFFs with the following receptor antagonists: Maraviroc (CCR5 antagonist, CAS 376348-65-1, Santa Cruz Biotechnology) at a concentration of 60nM in culture; AMD3100 (CXCR4 antagonist, CAS 155148-31-5, Santa Cruz Biotechnology), at concentration of 60nM in culture; RAP-310 (CCR5 antagonist,

Creative BioPeptides, Inc.) was used at a final concentration of 10 pM. Neurons were analyzed at DIV7.

Immunocytochemistry

For immunostaining, cells were fixed with 4% PFA in cytoskeleton preservation buffer (10 mM 2-(N-morpholino) ethanesulfonic acid (MES, Sigma-Aldrich, M3671) pH 6.1; 3 mM $MgCl_2$ (Merk, 1.05833.0250); 138 mM KCl (Merk, 529552); 2 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA, Sigma-Aldrich, E8145); 0.32 M sucrose (Merk, 1.07651.1000)), for 30 min at room temperature (RT). For cofilin-actin rods staining, neurons were permeabilized with cold (-20ºC) 100% methanol for 3 min at RT and blocked with 2.5% normal serum from donkey (Jackson ImmunoReasearch, 017-000-121) or goat (Sigma, 19H092) in 1% BSA/PBS for 1 h at RT. Next cells were incubated with primary antibodies: rabbit anti-T-cofilin 1:2000 (Bamburg lab, 1439 or Cell Signalling, 5175) and mouse anti-β3-tubulin 1:2000 (Promega, G7121) diluted in 1% BSA/PBS and incubated overnight at 4ºC. After washing, cells were incubated with secondary antibodies: donkey anti-mouse-Alexa Fluor 488 1:1000 (Invitrogen, A21202) or donkey anti-mouse-Alexa Fluor 647 1:1000 (Invitrogen, A31571) and donkey anti-rabbit–Alexa Fluor 568 1:1000 (Invitrogen, A10042) diluted in 1% BSA/PBS. Coverslips were mounted in Fluoromount-G (SouthernBiotech, 0100-01) or ProLong Diamond Antifade (ThermoFisher P36961). For αSyn staining, neurons were permeabilized with 2.5% triton X-100 in PBS for 20 min at RT and blocked with 5% normal donkey serum in 1% BSA/PBS for 1 h at RT. Subsequently, neurons were incubated with primary antibody: rabbit anti-αSyn pS129 1:1000 (Abcam, ab51253) diluted in 1% BSA/PBS and incubated overnight at 4ºC. After washing and incubation with secondary antibody: donkey anti-rabbit–Alexa Fluor 568 1:1000 (Invitrogen, A10042) diluted in 1% BSA/PBS, coverslips were mounted in Fluoromount-G.

In vivo αSyn PFFs injection

For αSyn PFFs stereotactic injection, mice were arbitrarily assigned to treatment groups at 8-months of age and euthanized at age 15-months. A microinjection syringe was inserted to target the substantia nigra pars compacta bilaterally (anterior–posterior, +/-3.0, medio-lateral, + 1.5, dorso-ventral, - 4.6 from bregma). Each injection delivered 10 µl of 5 µg/µL PFFs or, for controls, 10 µL of PBS. Post-surgical incisional pain was treated with bupivacaine and buprenorphine.

Immunohistochemistry

For tissue harvesting mice were perfused with PBS and brains were removed. Half of the brain was immersed in 4% paraformaldehyde in PBS for 48 hours, followed by immersion in 20% sucrose for 48 hours, and sectioned in a cryostat (40 μm thickness). For cofilin-actin rods staining sections were permeabilized with 100% methanol at -20ºC for 5 min at RT and blocked with 5% normal donkey serum in PBS for 1 h at RT, followed by incubation overnight at 4ºC with primary antibodies: rabbit anti-T-cofilin 1:1000 and mouse anti-β3-tubulin 1:1000 and subsequent washing and incubation with secondary antibodies: donkey anti-mouse-Alexa Fluor 488 1:500 and donkey anti-rabbit–Alexa Fluor 568 1:500 diluted in 1% BSA/PBS. Brain sections were then washed and rinsed in 70% ethanol and incubated with Sudan Black in 70% ethanol for 10 min at RT. Sections were washed and mounted in Ibidi mounting medium (Ibidi, 50001).

Imaging and quantifications

DIV7 Hippocampal neurons immunostained for cofilin were assessed for the presence of cofilin-actin rods in an upright epifluorescence microscope (Zeiss Axio Imager Z1, Carl Zeiss) at 40x magnification or on a Keyence Fluorescence Microscope with a 20x objective. The percentage of neurons with cofilin-actin rods was plotted.

DIV14 Hippocampal neurons immunostained for cofilin were imaged in an automated fluorescence widefield high-content screening microscope (IN Cell Analyzer 2000, GE Healthcare) at 40x magnification. Images were analyzed using Fiji software and the ratio between the number of rods and the total number of neurons analyzed was calculated and plotted as rod index (number of rods per number of neuronal nuclei).

Brain sections stained for cofilin were imaged in an automated fluorescence widefield high-content screening microscope (IN Cell Analyzer 2000, GE Healthcare) at 20x magnification. Images were stitched and the brain regions of interest analyzed using Fiji software and the results plotted as the number of rods per area.

Statistical analysis

All measurements were performed with the researcher blinded to the experimental condition when possible. Data are shown as mean ±SEM (standard error of the mean). Statistical significance was determined using the GraphPad Prism Software version 8 and the most appropriate statistic test being significance determined by *$p<0.05$, **$p<0.01$, **$p<0.001$ and ****$p<0.0001$. Statistic test and sample sizes are indicated in each figure legend.

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GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The overall life expectancy has increased over the years, and fortunately, several diseases have been progressively treated more effectively (Crimmins, 2015). Considering this, the prevalence of neurodegenerative disorders is likely to rise in the coming years and becomes imperative to provide an healthy aging brain and reduce severe disability in such patients (Rocca, 2018). This is a big challenge that scientists face these days, and this is a time when researchers must join efforts in order to find solutions in the most effective way possible.

An emerging link between actin cytoskeleton dysregulation and neurodegeneration has been proposed in the last years, making the cytoskeleton of particular relevance when studying the mechanisms underlying neurodegenerative disorders (Eira et al., 2016). Actin is a ubiquitous protein involved in several fundamental cellular processes, and its dysregulation can have tremendous implications for cellular functions, in general, and for neuronal functionality, in particular. In this regard, the current thesis contributes to expand the knowledge regarding actin cytoskeleton alterations in neurodegenerative disorders including Familial Amyloid Polyneuropathy and synucleinopathies.

Synucleinopathies constitute the second most common form of neurodegeneration in the world, which include LB dementias that account for the second most prevalent form of dementia after AD (Hansen et al., 2019). LB dementias are very devastating diseases as patients experience a severe decline in the quality of life and the care burden for the families increases significantly. These patients present cognitive impairment that consists of executive dysfunction, visual-spatial anomalies and abnormalities in memory (Sezgin et al., 2019). Due to the marked loss of acetylcholine neurons in patients with cognitive impairment in LB dementias, the use of cholinesterase inhibitors is in clinical practice with patients experiencing some degree of recovery (Meng et al., 2019). However, these remain symptomatic therapies without targeting or reversing neurodegeneration. Considering this, disease modifying therapies are a great need for these disorders.

The major pathological hallmark of these diseases is the accumulation of αSyn in the form of LBs, intracellularly, which triggers a cascade of events culminating in neuronal dysfunction and ultimately in neuronal death. One of the regions mostly affected by αSyn accumulation, that is related with cognitive impairment and dementia in these disorders, is the hippocampus (Adamowicz et al., 2017). However, the molecular mechanisms underlying hippocampal impairment are poorly explored. Here we showed that αSyn induces cofilin pathology in hippocampal neurons, which results from cofilin activation and is mediated by the PrP^C-NOX and the chemokine receptors CCR5 and CXCR4 pathways. Importantly, we found cofilin dysfunction not only in models of αSyn overexpression, but

also in models of exposure to αSyn PFFs, what suggests that the process of αSyn spreading and seeding might contribute to hippocampal cofilin pathology.

Cofilin pathology and specifically, cofilin-actin rods, have been studied in the context of hippocampal dysfunction in AD, in the process of neuronal damage in brain ischemia and more recently in *in vitro* models of HAND. Remarkably, in AD and HAND it was described a similar molecular pathway to the one reported in our study, proposing that different neurodegenerative disorders might have similar molecular mechanisms what could be helpful to find new therapeutic strategies. Interestingly, we found the same molecular pathways activated with different approaches and models, in which the overexpression of αSyn better represents a genetic scenario of the disease, while the exposure to αSyn PFFs most likely characterizes a sporadic form of the disease. This reinforces that, independently of the model used, there is a consistency of the pathways activated by αSyn pathology which invariably leads to cofilin dysregulation and rod formation.

Importantly, our *in vivo* data using the Thy1-aSyn mice showed a correlation between hippocampal cofilin pathology, synaptic defects and cognitive impairment, and one key future experiment should address the impact of modulating cofilin pathology *in vivo* for synaptic impairment and cognitive defects, to clearly demonstrate that targeting cofilin constitutes a novel therapeutic avenue for LB dementias. We have also showed rod formation in αSyn PFFs-injected mice which indicated that αSyn spreading and, most likely seeding, contributes to hippocampal cofilin pathology. In this respect, further studies may address if the αSyn PFFs-injected mouse model presents cognitive impairment, and if so, whether it correlates with the development of hippocampal cofilin pathology. Once these events are established, one should aim to target cofilin pathology and address the reversion or suppression of αSyn PFFs-induced hippocampal dysfunction and consequent behavior defects. Notably, synaptic dysfunction was described to precede neuronal death in both models, αSyn overexpression and αSyn PFFs administration, and we found cofilin pathology as a possible player in the process of synaptic dysregulation. Our findings are particularly relevant because cofilin is an essential protein for controlling actin cytoskeleton dynamics in synaptic structures, which is crucial for their proper function. Our results with the two distinct models suggest that, regardless of the model used, what seems to account for disease progression are increased levels of αSyn, which eventually reach the extracellular milieu, triggering αSyn spreading and seeding, what promotes cofilin pathology that contributes to hippocampal synaptic dysfunction.

Remarkably, one of the key experiments in the current work demonstrated that cofilin activation is required for rod formation and dendritic spine impairment caused by αSyn, what proposes that modulating cofilin activity might constitute a therapeutic approach.

Although we do not discard this option, we must consider that cofilin is a ubiquitous protein controlling actin dynamics in several cellular processes, thus finding an alternative strategy targeting cofilin pathology and cofilin-actin rods is imperative. Promising candidates to target cofilin pathology *in vivo* are the chemokine receptor antagonists as they demonstrated to be effective in inhibiting αSyn-induced rod formation in hippocampal neurons. Moreover, CCR5 antagonists have been proven to contribute to the amelioration of brain injury, stroke and HAND, pathologies that may share processes of neuroinflammation, synaptic impairment and cognitive decline, with AD and PD (Ndhlovu et al., 2014; Joy et al., 2019). Regarding synucleinopathies, future experiments should test CCR5/CXCR4 inhibitors *in vitro* and access whether their use rescues αSyn-induced dendritic spine impairment, and *in vivo* to address cofilin pathology (rod formation and cofilin phosphorylation), synaptic integrity and importantly, behavior, to evaluate their impact on cognitive functions. Considering that neuroinflammation is part of the spectrum of pathological processes occurring in synucleinopathies, the use of chemokine receptor inhibitors might also contribute to attenuate neuroinflammatory responses. In this regard, in a mouse model of AD, the use of a CCR5 antagonist DAPTA (D-Ala-peptide T-amide), resulted in decreased number of activated microglia and astrocytes as well as other parameters of neuroinflammation (Rosi et al., 2005). Additionally, a role for cofilin hyperactivation in the process of neuroinflammation has started to be unveiled (Alhadidi et al., 2018; Alhadidi and Shah, 2018). In this respect, it would be interesting to address the levels of microglia and astrocyte activation in the Thy1-αSyn mice and test whether the administration of the chemokine receptor inhibitors has also an impact in neuroinflammation in synucleinopathies. Importantly, the use of the most recent versions of chemokine receptor inhibitors, specifically the RAP-103, has several advantages when compared with the first generations of these inhibitors. RAP-103 represents a shorter analog of the clinical peptide DAPTA, it is orally available being this an enormous advantage when thinking on the therapeutic approach (Padi et al., 2012). Moreover, it has a rapid CNS entry, and it is potent, stable and has a long half-life (Padi et al., 2012). Considering all the properties, RAP-103 constitutes a promising therapeutic avenue.

Here, we found cofilin pathology as a new target for LB dementias, and corroborating our findings, a previous study reported the presence of cofilin-actin rods in the cingulate cortex of patients with neocortical Lewy Body pathology (Ordonez et al., 2018). Further studies should focus on the evaluation of cofilin-actin rods in several brain regions, including the hippocampus, in patients with LB dementias and also with pure motor Parkinson's disease (PD), to allow the confirmation of cofilin pathology as a cause of dementia and cognitive impairment associated with PD. In the future, a therapeutic approach targeting

cofilin pathology might be beneficial in protecting synaptic damage and hampering neurodegeneration in patients suffering from multiple dementias as is the case of not only LB dementias but also AD and HAND.

As a final remark, I consider that the current work strongly supports an involvement of cofilin pathology and cofilin-actin rods in the development of hippocampal dysfunction which might underlie cognitive impairment in LB dementias. The current thesis contributes to the understanding of the disease mechanisms and opens new lines of research in this field, which can hopefully contribute to the development of new therapeutic approaches in the future.

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